

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

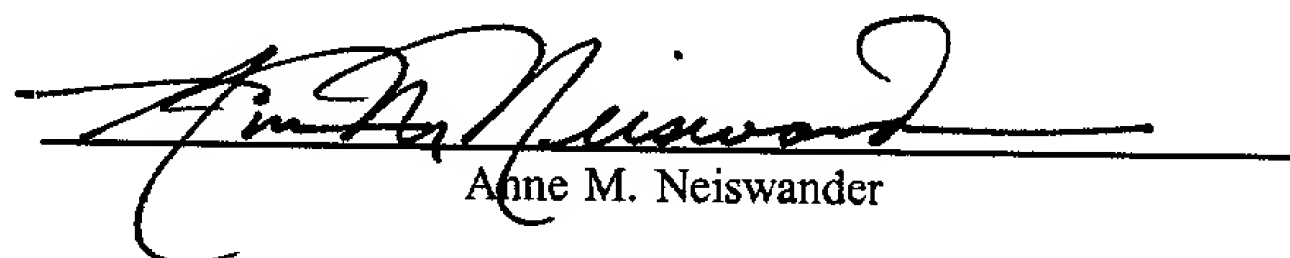
Box Patent Application
Assistant Commissioner For Patents
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is a Divisional application under 37 CFR § 1.53 (b) of pending patent application Serial No. 08/840,006, filed on 04/15/97, of inventors Tetsufumi Ueda and Eric D. Özkan, entitled **Compositions And Methods For The Inhibition Of Neurotransmitter Uptake Of Synaptic Vesicles**.

CERTIFICATION UNDER 37 C.F.R. § 1.10

I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the U.S. Postal Service on this date **July 10, 2000** in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number **EL 079 497 037 US** addressed to: **Box Patent Application**, Assistant Commissioner For Patents, Washington, D.C. 20231.


Anne M. Neiswander

1. Type Of Application

This new application is for a(n)

- ☒ Original (nonprovisional)
- ☒ Divisional application under 37 C.F.R. 1.53(b) of pending parent application Serial No. 08/840,006, filed on April 15, 1997, of inventors: Tetsufumi Ueda and Eric D. Özkan

2. Benefit Of Prior U.S. Application(s) (35 U.S.C. §§ 119(e), 120, or 121)

- ☒ The new application being transmitted claims the benefit of prior U.S. application(s) and enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

3. Papers Enclosed That Are Required For Filing Date Under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153 (Design) Application

- 39 Pages of Specification:
- 3 Pages of Claims:
- 1 Page of Abstract: and
- 16 Sheets of Formal Drawings.

4. Additional Papers Enclosed

- ☒ Preliminary Amendment
- ☒ Information Disclosure Statement (37 C.F.R. § 1.98), Form PTO-1449 (References sent in parent application)
- ☒ Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence (request under C.F.R. 1.821(e) to use the computer readable sequence listing in the parent application)
- ☒ Certificate Re: Sequence Listing

5. Declaration

- ☒ Enclosed.
- ☒ A copy of the executed declaration from the parent and of the amendments referred to therein is enclosed.

6. Assignment and Recordation Form

- ☒ Enclosed.
- ☒ The parent application was originally assigned of record to The Regents of the University of Michigan at Reel No. 9002, Frame No. 0783. A copy of the Assignment and Recordation Form from the parent and of the amendments referred to therein is enclosed.

7. Inventorship Statement

The inventorship for all the claims in this application is:

- ☒ the same

8. Language

- ☒ English

9. Fee Calculation (37 C.F.R. § 1.16)

☒ Regular application

CLAIMS AS FILED

Number Filed	Number Extra	Rate	Basic Fee - \$690.00 (37 C.F.R. § 1.16(a))
Total Claims (37 C.F.R. § 1.16(c))	16 - 20 =	0 × \$18.00 =	\$0.00
Independent Claims (37 C.F.R. § 1.16(b))	4 - 3 =	1 × \$78.00 =	\$78.00
Multiple Dependent Claim(s), if any (37 C.F.R. § 1.16(d))	+ \$260.00 =		\$0.00
Filing Fee Calculation			\$768.00

10. Small Entity Statement

☒ An executed Verified Statement that this filing is by a small entity has been filed in the parent application (copy enclosed) and such status is still proper and desired (37 C.F.R. §§ 1.28(a)).**Filing Fee Calculation (50% of above)** \$384.00

11. Fee Payment Being Made At This Time

☒ Enclosed☒ basic filing fee

\$384.00

Total Fees Enclosed \$384.00

12. Method of Payment of Fees

☒ Check in the amount of \$384.00

13. Authorization To Charge Additional Fees and Credit Overpayment

☒ The Commissioner is hereby authorized to charge payment of any fees associated with this communication or credit any overpayment to Deposit Account No.: 08-1290. An originally executed duplicate of this transmittal is enclosed for this purpose.

14. Power of Attorney by Assignee


☒ Enclosed.☒ A copy of the executed Power of Attorney document from the parent is enclosed.☒ Please address all further communications to:

Peter G. Carroll, Reg. No. 32,837

15. Return Receipt Postcard

☒ Enclosed

Dated: July 10, 2000


 Kamrin T. MacKnight
 Registration No.: 38,230

 MEDLEN & CARROLL, LLP
 220 Montgomery Street, Suite 2200
 San Francisco, California 94104
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☒ Incorporation By Reference Of Added Pages☒ Plus Added Pages For New Application Transmittal Where Benefit Of Prior U.S. Application(s) ClaimedNumber of pages added 1

**ADDED PAGES FOR APPLICATION TRANSMITTAL
WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED**

16. Relate Back

A. 35 U.S.C. § 120

☒ Amend the Specification by inserting before the first line, the sentence: "This is a Divisional of copending application Serial No. 08/840,006 filed on April 15, 1997."

17. Further Inventorship Statement Where Benefit Of Prior Application(s) Claimed

a. With respect to the parent copending U.S. Application from which this application claims benefit under 35 U.S.C. § 120, the inventors in this application are

☒ the same.

b. This inventorship for all the claims in this application is

☒ the same.

18. Notification In Parent Application Of This Filing

☒ A notification of the filing of this

☒ divisional

is being filed in the parent application from which this application claims priority under 35 U.S.C. § 120.

Applicant / Patentee: Tetsufumi Ueda *et al.*
For: **COMPOSITIONS AND METHODS FOR THE INHIBITION OF
NEUROTRANSMITTER UPTAKE OF SYNAPTIC VESICLES**

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR §§ 1.9(f) and 1.27(d) - NONPROFIT ORGANIZATION)**

I hereby declare that I am an official empowered to act on behalf of the university (or other institution of higher education) identified below:

THE REGENTS OF THE UNIVERSITY OF MICHIGAN
3003 South State Street, Ann Arbor, Michigan 48109-1280

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR § 1.9(e) for purposes of paying reduced fees under §§ 41(a) and (b) of Title 35, United States Code with regard to the invention entitled **COMPOSITIONS AND METHODS FOR THE INHIBITION OF NEUROTRANSMITTER UPTAKE OF SYNAPTIC VESICLES**, by inventors named **Tetsufumi Ueda *et al.***, described in the specification filed herewith.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR § 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Date: 6-16-97

By: 

Name: Robert L. Robb

Title: Director, Technology Management Office

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Tetsufumi Ueda *et al.*

Serial No.: To be assigned

Group No.: To be assigned

Filed: Herewith

Examiner: To be assigned

Entitled: **COMPOSITIONS AND METHODS FOR THE
INHIBITION OF NEUROTRANSMITTER
UPTAKE OF SYNAPTIC VESICLES**

PRELIMINARY AMENDMENT

Box PATENT APPLICATION

Assistant Commissioner for Patents

Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: July 10, 2000

By: 

Anne M. Neiswander

Sir:

This preliminary amendment places this Divisional Application of the prior application Serial No. 08/840,006 filed on April 15, 1997 (a copy of which is filed herewith) in condition to begin prosecution. A list of pending claims as they would appear following entry of this amendment is included as Appendix I for the Examiner's convenience. Also submitted herewith is a paper copy and a "Request Under 37 C.F.R. § 1.821(e) To Use Computer Readable Form From Another Application." The contents of the paper and computer readable copies are the same and include no new matter.

Please amend the prior application Serial No. 08/840,006 filed on April 15, 1997 as follows:

AMENDMENTS

IN THE SPECIFICATION

On page 1, line 1, after the title "**COMPOSITIONS AND METHODS FOR THE INHIBITION OF NEUROTRANSMITTER UPTAKE OF SYNAPTIC VESICLES**",

please insert --This is a Divisional of co-pending application Serial No. 08/840,006 filed on April 15, 1997.--

On page 3, line 27, after "amino acid sequence" please insert --(SEQ ID NO:1)--.

On page 4, line 11, after "amino acids" please insert --(SEQ ID NO:2)--.

On page 4, line 13, after "amino acids" please insert --(SEQ ID NO:3)--.

On page 4, line 16, after "amino acids" please insert --(SEQ ID NO:4)--.

On page 6, line 24, after "IPF α sequence" please insert --(SEQ ID NO:5)--.

On page 6, line 25, after "predicted sequence" please insert --(SEQ ID NO:6)--.

On page 33, line 28, after "Figure 6A" please insert --(SEQ ID NO:5)--.

On page 34, line 8, after "Figure 6B" please insert --(SEQ ID NO:6)--.

On page 40, line 8, after "amino acid sequence" please insert --(SEQ ID NO:1)--.

On page 40, line 17, after "amino acids" please insert --(SEQ ID NO:3)--.

On page 40, line 19, after "amino acids" please insert --(SEQ ID NO:4)--.

Please insert the attached Sequence Listing as new pages --40-49--.

IN THE CLAIMS:

Please renumber the Claims pages from pages "40-42" to --50-52--.

Please cancel Claims 1-14.

Please amend Claims 15 and 19 as follows:

15. (Once amended) A method for assessing overcoming synaptic vesicle glutamate uptake inhibition activity, comprising:

- a) providing:
 - i) synaptic vesicles,
 - ii) [as] a composition comprising purified fodrin fragment having glutamate uptake inhibition activity, said fragment having an N-terminus and a C-terminus, and
 - iii) candidate compound; and
- b) combining said candidate compound with said synaptic vesicles and said fragment such that the effect of said candidate compound on glutamate uptake by said synaptic vesicles can be assessed.

19. (Once Amended) A method for assessing overcoming synaptic vesicle glutamate uptake inhibition activity, comprising:
- a) providing:
 - i) synaptic vesicles,
 - ii) a composition comprising purified fragment of IPF having synaptic vesicle glutamate uptake inhibition activity, said fragment having an N-terminus and a C-terminus, and
 - iii) candidate compound; and
 - b) combining said candidate compound with said synaptic vesicles and said purified fragment such that the effect of said candidate compound on said fragment's effect on glutamate uptake by said synaptic vesicles can be assessed.

Please add Claims 23-30 as follows:

23. (New) The method of Claim 15, wherein said N-terminus is Tyr²⁶ of fodrin.
24. (New) The method of Claim 15, wherein said purified fragment comprises a fragment of IPF α .
25. (New) The method of Claim 19, wherein said N-terminus is Tyr²⁶ of fodrin.
26. (New) The method of Claim 19, wherein said purified fragment comprises a fragment of IPF α .
27. (New) A method for assessing overcoming synaptic vesicle glutamate uptake inhibition activity, comprising:
- a) providing:
 - i) synaptic vesicles,
 - ii) a composition comprising preferred fragment of fodrin having glutamate uptake inhibition activity, said fragment having an N-terminus and a C-terminus, wherein said purified fragment comprises a peptide having the amino

acid sequence EAALTSEEVG within 150 amino acids of the C-terminus of the peptide, and

- iii) candidate compound; and
- b) combining said candidate compound with said synaptic vesicles and said fragment such that the effect of said candidate compound on glutamate uptake by said synaptic vesicles can be assessed.

28. (New) A method for assessing overcoming synaptic vesicle glutamate uptake inhibition activity, comprising:

- a) providing:
 - i) synaptic vesicles,
 - ii) a composition comprising purified peptide having glutamate uptake inhibition activity with an N-terminus sequence comprising the amino acid sequence YHRFK, and
 - iii) candidate compound; and
- b) combining said candidate compound with said synaptic vesicles and said fragment such that the effect of said candidate compound on glutamate uptake by said synaptic vesicles can be assessed.

29. (New) The method of Claim 28, wherein said purified peptide has an N-terminus comprising the amino acid sequence YHRFKELSTL.

30. (New) The method of Claim 29, wherein said purified peptide has an N-terminus comprising the amino acid sequence YHRFKELSTLRRQKLEDSYR.

IN THE ABSTRACT:

Please renumber the Abstract page from page "43" to --53--.

R E M A R K S

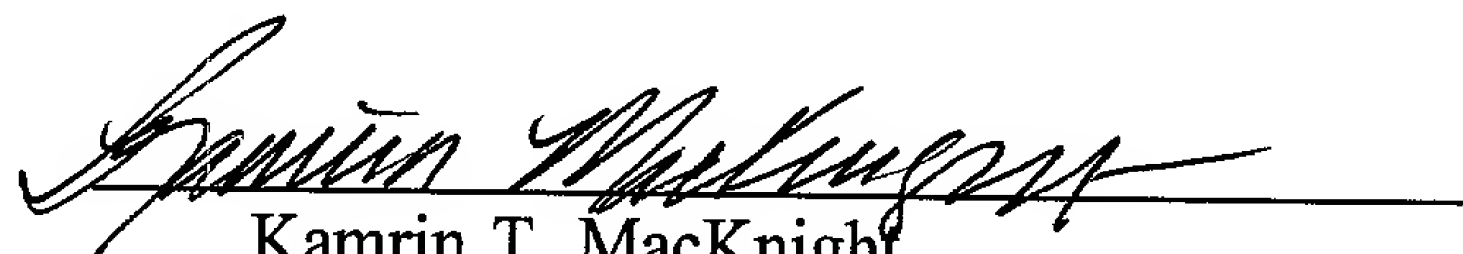
Applicants respectfully request entry of the above preliminary amendment.

Pursuant to 35 U.S.C. § 120, the specification has been amended to recite the divisional status of this application, and its relationship to a previous application filed by Applicants. The specification has also been amended to add sequence identifiers and to include a paper copy of the Sequence Listing in conformance with 37 C.F.R. §§ 1.821-1.825. Applicants' amendments do not introduce new matter.

This application presents for prosecution Claims 15-22 which were designated as Group IV in the restriction requirement which was mailed on May 11, 1998 in the parent application Serial No. 08/840,006. Claims 1-10 (Groups I and II) have been cancelled as they are currently being prosecuted in co-pending application No. 08/840,006. Claims 11-14 (Group III) have been cancelled without prejudice to their prosecution in another application. Claims 23-30 have been added to more clearly describe Applicants' invention.

Respectfully submitted,

Dated: July 10, 2000


Kamrin T. MacKnight
Registration No. 38,230

Please direct all communications to:

Peter G. Carroll
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COMPOSITIONS AND METHODS FOR THE INHIBITION OF NEUROTRANSMITTER UPTAKE OF SYNAPTIC VESICLES

This invention was made with government support under Javits Neuroscience Investigator Award NS 26884 awarded by the National Institutes of Health,
5 Department of Health and Human Services. The Government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention pertains to compositions and methods for the inhibition of neurotransmitter uptake of synaptic vesicles.

10 BACKGROUND

Glutamate is now widely accepted as the major excitatory neurotransmitter in the central nervous system of all vertebrates [see *e.g.*, Nakanishi (1992) *Science* 258, 597-603]. Abnormalities in glutamatergic synaptic transmission have been implicated in many neuropathologies, including certain seizures, ischemia-induced neuronal death,
15 schizophrenia, Alzheimer's disease, Parkinson's disease, and Huntington's disease [see *e.g.*, Coyle and Puttfarcken (1993) *Science* 262, 689-695].

Excessive release of glutamate into the synaptic cleft is believed to be a common underlying basis for many of these disease states. There is also evidence that some glutamate receptors such as the NMDA (N-methyl-D-aspartate) and the
20 metabotropic receptors may be involved in neuronal plasticity [see *e.g.*, Bashir *et al.* (1993) *Nature* 363, 347-350].

Evidence which has been accumulated for the last decade strongly supports the notion that glutamatergic neurotransmission occurs via an exocytotic process involving the interaction of glutamate-containing synaptic vesicles with the plasma membrane of
25 the presynaptic ending. In support of this is the observation that glutamate is taken up into purified, isolated synaptic vesicles in an ATP-dependent manner [see *e.g.*, Naito and Ueda (1983) *J. Biol. Chem.* 258, 696-699; and Tabb and Ueda (1991) *J. Neurosci.*

11, 1822-1828], consistent with immunocytochemical evidence that glutamate is concentrated in synaptic vesicles which are distinct from GABA-vesicles [Storm-Mathisen *et al.* (1983) *Nature* 301, 517-520].

5 Biochemical evidence also suggested that high concentrations of glutamate are accumulated in brain synaptic vesicles *in vivo*. Studies by Nicholls and co-workers indicate that the exocytotic pool of glutamate originates from a noncytoplasmic site within the nerve terminal [see *e.g.*, Nicholls and Sihra (1986) *Nature* 321, 772-773; and McMahon and Nicholls (1991) *Biochim. Biophys. Acta* 1059, 243-264].

10 Moreover, Kish and Ueda [(1991) *Neurosci. Lett.* 122, 179-182] provided evidence that vesicular glutamate is released in a calcium-dependent manner from permeabilized synaptosomes. This body of evidence clearly demonstrates that synaptic vesicles are the storage site of the glutamate to be released from nerve terminals.

15 The vesicular glutamate uptake system has several distinctive properties which distinguish it from the cellular glutamate re-uptake system present in the plasma membrane. It is stringently specific for glutamate, has a relatively high K_m , and requires low concentrations of chloride for optimal activity [(Naito and Ueda (1985) *J. Neurochem.* 44, 99-109; and Fykse *et al.* (1989) *J. Neurochem.* 52, 946-951].

20 The driving force for glutamate uptake is provided by an electrochemical proton gradient generated by a V-type H^+ -ATPase in the synaptic vesicle membrane [Naito and Ueda (1985) *J. Neurochem.* 44, 99-109]. The precise mechanism by which the glutamate transporter utilizes this proton gradient to drive glutamate uptake is not fully understood; however, compounds that interfere with the formation of such gradients have a marked inhibitory effect on glutamate transport [Naito and Ueda (1985) *J. Neurochem.* 44, 99-109; Tabb *et al.* (1992) *J. Biol Chem* 267, 15412-15418].

25 It has been proposed that glutamate uptake into synaptic vesicles represents the critical step in diverting glutamate away from the metabolic pathway and toward the neurotransmitter pathway [Ueda (1986) in *Excitatory Amino Acids* (Roberts P.J., Storm-Mathisen J., and Bradford H.F., eds), pp. 173-195, Macmillan Press, London]. Thus, it is desirable to regulate the vesicular glutamate uptake system under normal

physiological conditions. Alterations in such a regulatory system could cause the abnormalities in glutamatergic neurotransmission implicated in the variety of central nervous system disorders mentioned above. However, while many studies have focused on changes associated with postsynaptic glutamate receptors, few have addressed presynaptic regulation of glutamatergic neurotransmission at the level of vesicular transport. What is needed are compositions and methods for regulating the uptake of glutamate into the synaptic vesicles.

SUMMARY OF THE INVENTION

The invention provides compositions and methods for the inhibition of neurotransmitter uptake of synaptic vesicles. In one embodiment, the present invention provides compositions and methods for regulating the uptake of glutamate by synaptic vesicles. In one embodiment, the present invention contemplates the inhibition of glutamate uptake by synaptic vesicles with an inhibitor. In one embodiment, the inhibitor is a peptide, and in a preferred embodiment, the peptide is inhibitory protein factor (IPF).

The term "IPF" is used in reference to three proteins (IPF $\alpha\beta\gamma$) each having an apparent molecular weight on electrophoresis gels of between 130 and 138 kD. IPF can be purified from animal brain tissue, including human tissue.

In one embodiment, the present invention contemplates a composition comprising a purified fragment of fodrin having glutamate uptake inhibition activity, said fragment having an N-terminus and a C-terminus. The composition is not limited by the exact amino acid sequence, however in one embodiment the N-terminus of the fragment corresponds to Tyr²⁶ of fodrin (that is to say, the N-terminus of the fragment begins with this amino acid and has additional amino acids that follow after this initial amino acid in the sequence order found in intact fodrin - although it is not intended to be limited to any precise length). In another embodiment, the purified fragment comprises a peptide having the amino acid sequence EAALTSEEVG within 150 amino

acids of the C-terminus of the peptide, more preferably, within 130 amino acids of the C-terminus of the peptide, and most preferably, within 120 amino acids of the C-terminus of the peptide.

Likewise, the present invention is not limited by the precise size of the fragment of fodrin. In one embodiment, the purified fragment comprises IPF α , while in other embodiments the composition comprises a purified fragment of IPF α . In yet other embodiments, the purified fragment comprises IPF β or IPF γ .

While the present invention is not limited to a specific amino acid sequence, in one embodiment the present invention contemplates a composition comprising a purified peptide having glutamate uptake inhibition activity with an N-terminus sequence comprising the amino acids YHRFK (*i.e.*, the peptide N-terminus begins with this sequence and has at least these amino acids in this order). In another embodiment, the said purified peptide has an N-terminus comprising the amino acids YHRFKELSTL (*i.e.*, the peptide N-terminus begins with this sequence and has at least these amino acids in this order). In yet another embodiment, the purified peptide has an N-terminus comprising the amino acids YHRFKELSTLRRQKLEDSYR (*i.e.*, the peptide N-terminus begins with this sequence and has at least these amino acids in this order).

The present invention also contemplates a method of isolating a glutamate uptake inhibitor, comprising: a) providing an animal brain; and b) subjecting said animal brain to a purification procedure such that a purified fodrin fragment having glutamate uptake inhibition activity is produced. The present invention is not limited by the specific purified fragment. In certain embodiments the purified fragment comprises IPF α , IPF β or IPF γ .

The present invention contemplates screening assays for candidate compounds. While the present invention is not limited by any particular screening assay, in one embodiment, the present invention contemplates a method for testing compounds for their ability to overcome or offset the synaptic vesicle glutamate uptake inhibition activity of the fodrin fragments of the present invention. In one embodiment, the screening method comprises a) providing: i) synaptic vesicles, ii) as purified fodrin

fragment having glutamate uptake inhibition activity, and iii) candidate compound; and
b) combining said candidate compound with said synaptic vesicles and said fragment
such that the effect of said candidate compound on glutamate uptake by said synaptic
vesicles can be assessed. The method is not limited by the purified fragment utilized,
5 in certain embodiments the purified fragment comprises IPF α , IPF β or IPF γ .

Another screening assay contemplated by the present invention contemplates a
method for testing compounds for their ability to overcome or offset the synaptic
vesicle glutamate uptake inhibition activity of fragments of IPF. In one embodiment,
the method comprises a) providing: i) synaptic vesicles, ii) a purified fragment of IPF
10 having synaptic vesicle glutamate uptake inhibition activity, and iii) candidate
compound; and b) combining said candidate compound with said synaptic vesicles and
said purified fragment such that the effect of said candidate compound on said
fragment's effect on glutamate uptake by said synaptic vesicles can be assessed. The
assay is not limited by the nature of the purified fragments, however in certain
15 embodiments the purified fragment comprises fragments of IPF α , IPF β or IPF γ .

The present invention also contemplates screening method of testing candidate
compound for inhibition of calpain cleavage of fodrin, comprising: a) providing: i)
fodrin, ii) calpain, and iii) candidate compound; and b) combining said candidate
compound with said fodrin and said calpain such that the effect of said candidate
20 compound on calpain cleavage of fodrin can be assessed. In one embodiment,
antibodies to fodrin are used as control inhibitors of calpain digestion.

In yet another embodiment, the present invention contemplates a screening
method of testing candidate compound for inhibition of trypsin cleavage of IPF,
comprising: a) providing: i) trypsin, ii) purified IPF, and iii) candidate compound; and
25 b) combining said candidate compound with said trypsin and said IPF such that the
effect of said candidate compound on cleavage of said IPF by said trypsin can be
assessed. In one embodiment, antibodies to IPF α fragments are used as control
inhibitors of trypsin cleavage. The screening method is not limited by the nature of
the purified IPF utilized. In certain embodiments, the purified fragment comprises IPF
30 α , IPF β or IPF γ .

The present invention also contemplates antibodies to fodrin and purified fragments of fodrin. While the present invention is not limited by the sequence of the epitope of the purified fragment, in one embodiment the epitope sequence corresponds to a portion of a decapeptide from fodrin. In yet another embodiment the antibodies have been passed through a column containing purified fodrin. In still another embodiment, the antibodies will bind to a purified fragment of fodrin, but do not bind to fodrin.

The present invention also contemplates immobilized fodrin and fragments, such as fragments bound to a resin. The present invention is not limited by the specific nature of the purified fragment bound. In certain embodiments the purified fragment comprises IPF α , IPF β or IPF γ . In yet another embodiment, the purified fragment comprises a fragment of IPF α .

DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a series of halogenated glutamic acid analogs useful for inhibiting synaptic vesicle glutamate uptake.

Figure 2 depicts the SDS-PAGE profiles of fractions obtained during the purification of IPF.

Figure 3 compares the SDS-PAGE profile (lower panel) of peak inhibitory fractions as eluted from Mono Q HPLC (upper panel).

Figure 4 depicts a dose-response curve for purified IPF α . As the concentration of IPF is increased, the ATP-dependent glutamate uptake is decreased.

Figure 5 graphically depicts the specificity of the inhibitory effect produced by IPF α .

Figure 6A compares partial IPF α sequence with the sequence of human α fodrin, while **Figure 6B** shows the predicted sequence of IPF α based upon the C-terminus being commensurate with the calpain cleavage site for fodrin and the N-terminus beginning at amino acid 26 of fodrin.

Figure 7 shows the effect of trypsin exposure on IPF. Lane A shows the SDS-PAGE profile of the partially purified IPF composition without trypsin treatment.

Lane B shows the SDS-PAGE profile of the partially purified IPF composition with trypsin treatment. It is clear that the 138 kD IPF band is not present, demonstrating the digestion of IPF by trypsin. Lane C shows the SDS-PAGE profile of the partially purified IPF composition in the presence of trypsin and pancreatic trypsin inhibitor.

5 Figure 8 depicts the results of the fractions obtained from Figure 7. Lane A depicts control glutamate uptake without additives. Lane B depicts glutamate uptake by synaptic vesicles in the presence of trypsin and pancreatic trypsin inhibitor. Lane C depicts glutamate uptake by synaptic vesicles in the presence of partially purified IPF. Lane D depicts glutamate uptake by synaptic vesicles in the presence of partially
10 purified IPF and trypsin. Lane E depicts glutamate uptake by synaptic vesicles in the presence of partially purified IPF, trypsin and pancreatic trypsin inhibitor.

Figure 9 depicts the results of Western Blot assays of antibodies raised to a fodrin decapeptide. Panel A demonstrates the binding of antibodies to both fodrin and IPF, while Panel B demonstrates binding of antibodies to IPF without binding to
15 fodrin.

DEFINITIONS

"Glutamate uptake inhibition activity" as used herein refers to the property of inhibiting the uptake of glutamate by synaptic vesicles. A "glutamate uptake inhibitor" is a peptide that demonstrates glutamate uptake inhibition activity.

20 "IPF" as used herein refers to a peptide derived from animal brain tissue that is a glutamate uptake inhibitor. IPF is a designation for three similar peptides "IPF $\alpha\beta\gamma$."

"Neurosynaptic disorder" as used herein refers to undesirable neuronal activity resulting in seizures of a subject or damage to a subject's neural tissue. Examples of
25 such conditions include, but are not limited to, epileptic seizures, Huntington's Disease, Alzheimer's Disease, *etc.*

"GABA" as used herein refers to γ -aminobutyric acid and acts as an inhibitory transmitter in the central nervous system.

"Fodrin" as used herein refers to a protein of a family of proteins that bundle and crosslink actin filaments. Actin filaments are a part of the cytoskeleton and are responsible for maintaining cell structure and integrity. Fodrin is a rod-shaped protein that lines the cortical cytoplasm of neurons. Fodrin can be identified as a high molecular weight protein present in brain membranes by i) comigration on NaDodSO₄ polyacrylamide gels with purified fodrin, ii) reactivity with antibodies to purified fodrin, and iii) a proteolytic map following calpain activation comparable to that found after calpain mediated degradation of pure fodrin. "α fodrin" as used herein refers to the fodrin as isolated from human cells. "Fodrin fragment" as used herein refers to a peptide or protein whose amino acid sequence is equivalent to a portion of the amino acid sequence of fodrin. "Purified fodrin fragment" as used herein refers to a fodrin fragment that is isolated from the natural environment of fodrin or a fodrin fragment created by degradation of fodrin that has been isolated from its natural environment.

"Calpain" as used herein refers to a calcium calmodulin dependent neutral proteinase isolated from the cytosolic fractions of various animal tissues or cells with a molecular weight of 94-100 kDa by gel filtration on Sephacryl 300.

"Trypsin" as used herein refers to a proteolytic enzyme from pancreatic juice that hydrolyses polypeptides on the carboxyl side of arginine and lysine residues.

"Trypsin sensitive" as used herein refers to a peptide with an arginine or lysine residue that is sensitive to cleavage by trypsin on the carboxyl side.

"Purified" as used herein refers to a composition wherein at least one component has been removed from the crude extract causing the proportion of protein of interest (*e.g.*, IPF or fodrin) to be increased relative to the total proteins found in a crude extract.

"Apparent molecular weight" as used herein refers to the estimated molecular weight as determined by methods (*e.g.*, gel electrophoresis, sucrose gradient, gel filtration) and compared to the molecular weights of standards run by the same method.

"True molecular weight" as used herein refers to the molecular weight of a peptide as determined by the additive molecular weights of its component amino acids as determined by complete sequencing of the peptide.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention pertains to compositions and methods for the inhibition of neurotransmitter uptake of synaptic vesicles. In a preferred embodiment, the inhibitor is IPF. In another embodiment, the inhibitor is a product of exposure of IPF to trypsin.

10 IPF is isolated from brain tissue and refers to three distinct proteins with relative molecular weights of 138,000 (α), 135,000 (β), and 132,000 (γ), respectively. It has been determined that IPF is best purified from brain material excised from young animals as opposed to mature animals.

15 While an understanding of the precise mechanism is not necessary to carry out the present invention, it is believed that IPF interacts with a synaptic vesicle-specific protein that leads to a blockade of neurotransmitter storage by an indirect mechanism. The physiological role of IPF remains to be elucidated. The fact that IPF seems to derive from α fodrin is particularly intriguing since fodrin purified from whole brain is itself devoid of inhibitory activity (data not shown). Regardless of the mechanism of action of IPF, it is a potent, endogenous inhibitor of vesicular neurotransmitter uptake.

20 The present invention also contemplates degradation products of IPF that exhibit synaptic vesicle glutamate uptake inhibition and the use thereof. While the present invention is not limited by the type or method of producing these degradation products, in one embodiment IPF is degraded with trypsin and these degradation products inhibit glutamate uptake by synaptic vesicles.

25 Mimetics

 Compounds mimicking the necessary conformation for recognition and docking to the receptor binding to the peptides of the present invention are contemplated as within the scope of this invention. For example, mimetics of IPF peptides are

contemplated. A variety of designs for such mimetics are possible. For example, cyclic IPF-containing peptides, in which the necessary conformation for binding is stabilized by nonpeptides, are specifically contemplated. United States Patent No. 5,192,746 to Lobl *et al*, United States Patent No. 5,169,862 to Burke, Jr., *et al*, United States Patent No. 5,539,085 to Bischoff *et al*, United States Patent No. 5,576,423 to Aversa *et al*, United States Patent No. 5,051,448 to Shashoua, and United States Patent No. 5,559,103 to Gaeta *et al*, all hereby incorporated by reference, describe multiple methods for creating such compounds.

Synthesis of nonpeptide compounds that mimic peptide sequences is also known in the art. Eldred *et al*, (*J. Med. Chem.* 37:3882 (1994)) describe nonpeptide antagonists that mimic the Arg-Gly-Asp sequence. Likewise, Ku *et al*, (*J. Med. Chem.* 38:9 (1995)) give further elucidation of the synthesis of a series of such compounds. Such nonpeptide compounds that mimic IPF peptides are specifically contemplated by the present invention.

The present invention also contemplates synthetic mimicking compounds that are multimeric compounds that repeat the relevant peptide sequence. As is known in the art, peptides can be synthesized by linking an amino group to a carboxyl group that has been activated by reaction with a coupling agent, such as dicyclohexylcarbodiimide (DCC). The attack of a free amino group on the activated carboxyl leads to the formation of a peptide bond and the release of dicyclohexylurea. It can be necessary to protect potentially reactive groups other than the amino and carboxyl groups intended to react. For example, the α -amino group of the component containing the activated carboxyl group can be blocked with a tertbutoxycarbonyl group. This protecting group can be subsequently removed by exposing the peptide to dilute acid, which leaves peptide bonds intact.

With this method, peptides can be readily synthesized by a solid phase method by adding amino acids stepwise to a growing peptide chain that is linked to an insoluble matrix, such as polystyrene beads. The carboxyl-terminal amino acid (with an amino protecting group) of the desired peptide sequence is first anchored to the

polystyrene beads. The protecting group of the amino acid is then removed. The next amino acid (with the protecting group) is added with the coupling agent. This is followed by a washing cycle. The cycle is repeated as necessary.

In one embodiment, the mimetics of the present invention are peptides having sequence homology to the above-described IPF peptides. One common methodology for evaluating sequence homology, and more importantly statistically significant similarities, is to use a Monte Carlo analysis using an algorithm written by Lipman and Pearson to obtain a Z value. According to this analysis, a Z value greater than 6 indicates probable significance, and a Z value greater than 10 is considered to be statistically significant. W.R. Pearson and D.J. Lipman, Proc. Natl. Acad. Sci. (USA), 85:2444-2448 (1988); D.J. Lipman and W.R. Pearson, Science, 227:1435-1441 (1985). In the present invention, synthetic polypeptides useful in glutamate uptake inhibition by synaptic vesicles are those peptides with statistically significant sequence homology and similarity (Z value of Lipman and Pearson algorithm in Monte Carlo analysis exceeding 6).

Glutamate Analogs as Inhibitors of Glutamate Uptake in Synaptic Vesicles

The present invention contemplates the use of glutamate analogs to inhibit glutamate uptake by synaptic vesicles. While not being limited by the nature of the glutamate analogs, halogenated analogs of glutamic acid and cyclic analogs of glutamate are contemplated.

While the present invention is not limited by the type of halogenated glutamic acid analog utilized, examples of such compounds are set forth in Figure 1. Where the structures set forth signify an X substituent, any of the halogens (*i.e.*, fluorine, chlorine, bromine or iodine) may be used.

For cyclic analogs of glutamate, it is contemplated that compounds that have a three-dimensional charge distribution identical or very close to that of (1S,3R)-1-aminocyclopentane-1,3-dicarboxylate (ACPD), an analog of L-glutamate, are useful. Preferably, the compound has a hydrophobic moiety similar to that seen in (1S,3R)-ACPD. Examples include, but are not limited to, 1-aminocyclobutane-1,3-dicarboxylic

acid, 1-aminocyclohexane-1,3-dicarboxylic acid and 1-aminocycloheptane-1,3-dicarboxylic acid [all available commercially from Tocris-Cookson, Bristol, UK]. The present invention is not limited by the form of the cyclic analog of glutamate. For example, derivatives of cyclic analogs (*e.g.*, halogenated, methylated or ethylated) are also contemplated.

Drug Screening Assays

The present invention contemplates *in vitro* screening assays for the discovery of 1) new glutamate uptake inhibitors, 2) compounds that can overcome glutamate uptake inhibition, 3) inhibitors of calpain degradation of fodrin, 4) inhibitors of trypsin degradation of IPF and 5) compounds that can overcome glutamate uptake inhibitor by the trypsin degradation products of IPF. The present invention also contemplates *in vivo* screening assays to assess efficacy.

These screening assays are described in detail below. Glutamate and synaptic vesicles are utilized in the *in vitro* assays. The present invention is not limited by the nature of the synaptic vesicles utilized. For example, synaptic vesicles from bovine and mice [as set forth in Examples below] and from rats [Carlson *et al.* (1989) *J. Neurochemistry* 53 1889-1894], as well as other sources are contemplated.

1. New Glutamate Uptake Inhibitors

The present invention contemplates the use of IPF with glutamate and synaptic vesicles to assess glutamate uptake inhibition of other candidate inhibitors. While the present invention is not limited by the actual assay protocol, IPF can be used as a standard for testing the inhibition properties of other candidate compounds.

In such an assay, glutamate uptake inhibition by synaptic vesicles is assessed as set forth in the Examples below. Such uptake can be measured with and without the presence of IPF. Meanwhile, candidate inhibitor can be assayed under similar conditions in the absence of IPF. The extent of glutamate uptake by the synaptic vesicles in the presence of candidate inhibitor can then be compared to uptake with no

additive and uptake in the presence of IPF. If uptake in the presence of candidate inhibitor reduces glutamate uptake by synaptic vesicles as compared to uptake in the absence of candidate inhibitor, the candidate inhibitor is considered to exhibit glutamate uptake inhibition activity.

2. Compounds That Overcome Glutamate Uptake Inhibition

The present invention contemplates the screening of compounds useful for overcoming inhibition of glutamate uptake by synaptic vesicles. While the present invention is not limited by the actual assay protocol, it is contemplated that candidate compounds can be screened for their ability to overcome IPF inhibition of glutamate uptake by synaptic vesicles.

In such an assay, glutamate uptake by synaptic vesicles is inhibited by IPF as set forth in the Examples below. Candidate compound can be added and uptake by synaptic vesicles can be assessed. If glutamate uptake by synaptic vesicles in the presence of IPF and candidate compound is increased in relation to uptake in the presence of IPF alone, the candidate compound overcomes glutamate uptake inhibition activity by synaptic vesicle glutamate uptake inhibitors.

3. Inhibitors of Calpain Degradation of Fodrin

It is believed that the C-terminus of IPF corresponds with a calpain cleavage site of fodrin. It is therefore hypothesized that the C-terminus region of IPF can be formed from fodrin by cleavage with calpain. This cleavage should result in a fragment of fodrin of about 150 kD, with a C-terminus matching that of IPF and an N-terminus similar to that of fodrin. This fragment is in contrast to IPF itself of about 138 kD, whose N-terminus corresponds with the amino acid sequence of fodrin beginning at position 26 (*i.e.*, lacking the first 25 amino acids of fodrin).

The present invention contemplates the screening of compounds useful for inhibiting calpain cleavage of fodrin. While the present invention is not limited by the actual assay protocol, it is contemplated that candidate compounds can be screened for

their ability to inhibit calpain cleavage of fodrin by introducing candidate compound with fodrin and calpain, and assessing the presence and nature of any cleavage products (*e.g.*, by examining the products on gel electrophoresis).

In such an assay, if the cleavage products in the presence of candidate compound do not correspond with the 150 kD or 138 kD proteins described above, the candidate compounds is useful as a inhibitor of fodrin cleavage by calpain.

4. Inhibitors of Trypsin Degradation of IPF

As set forth in the Examples below, it has been determined that trypsin can cleave IPF. These degradation products have synaptic vesicle glutamate uptake inhibition activity similar to that of IPF itself.

The present invention contemplates the screening of compounds useful for inhibiting trypsin degradation of IPF. While the present invention is not limited by the actual assay protocol, it is contemplated that candidate compounds can be screened for their ability to inhibit trypsin cleavage of IPF by introducing candidate compound with IPF and trypsin, and assessing the presence and nature of any cleavage products.

In such an assay, if the cleavage products in the presence of candidate compound do not correspond with the trypsin cleavage products described above, the candidate compounds is useful as a inhibitor of IPF cleavage by trypsin.

5. Overcoming Glutamate Uptake Inhibition by IPF Fragments

As described above, fragments of IPF exhibit synaptic vesicle glutamate uptake inhibition activity. While the present invention is not limited by the nature of the fragments, the present invention does contemplate the screening of compounds useful for overcoming such IPF fragment inhibition of glutamate uptake by synaptic vesicles.

In such an assay, glutamate uptake by synaptic vesicles is inhibited by IPF fragments as set forth in the Examples below. Candidate compound can be added and uptake by synaptic vesicles can be assessed. If glutamate uptake by synaptic vesicles

in the presence of IPF fragments and candidate compound is increased in relation to uptake in the presence of IPF fragments alone, the candidate compound overcomes glutamate uptake inhibition activity by IPF fragments.

6. *In vivo* Assays of Glutamate Uptake Inhibitors

5 The present invention contemplates the treatment of synaptic disorder in subjects and is not limited by the type of glutamate uptake inhibitor utilized. Candidate inhibitors may be tested prior to administration to a patient. Once drugs possessing inhibitory activity and lacking neuronal toxicity are identified, they may be administered according to the invention to a patient exhibiting symptoms of a disease
10 or disorder associated with neuronal injury or death, particularly to patients in which neuronal injury or death is a result of glutamate toxicity. Preferred inhibitors are those which are capable of crossing the blood/brain barrier.

 The above-identified inhibitors and also drugs identified according to the invention as inhibitors of glutamate uptake by synaptic vesicles can be tested in
15 animals for their effectiveness in inhibiting or preventing neuronal seizures and injury and/or death. Conventional animal testing systems are well-known in the art. Each animal may be injected with a range of doses of the drug and the toxicity or lack of toxicity of the drug may be assessed by the survival rate of the animals. Animals which exhibit symptoms of neuronal injury, or animals subject to conditions generating cerebral infarction (*i.e.*, stroke), e.g, oxygen or glucose deprived animals, can then be
20 tested. The effects of the drug on peripheral tissues can be assessed by histological examination of the tissues. Based on these *in vivo* tests, the overall effectiveness of the drug can be determined, and an effective dose and mode of administration can also be determined. The present invention is not limited by the nature of the animal or
25 disease modeled. In one embodiment, the animal model is of a type as set forth in the Examples below (*e.g.*, the EL mouse strain).

Drug Therapy and Formulations

The present invention is not limited by the method of administration of the inhibitor. In one embodiment, it is by conventional means available for use in conjunction with pharmaceuticals; either in combination with one another or in combination with other therapeutic agents. It is contemplated that the methods of the present invention be administered alone or can be administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice. In one embodiment, the inhibitor is administered by a implanting device for the release of neuroinhibitory compounds. One example of such a device is described in U.S. Patent No. 5,573,528 to Aebischer *et al.*, herein incorporated by reference.

In one preferred embodiment, the inhibitor is administered orally in solid dosage forms, such as capsules, tablets, or powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions; however, it can also be administered parenterally, in sterile liquid dosage forms, or rectally in the form of suppositories.

One skilled in the art will be capable of adjusting the administered dose depending upon known factors such as the mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effect desired. In one embodiment, the dosage is increased to overcome a non-responsive condition.

Additionally, the inhibitor can be employed in admixture with conventional excipient, *i.e.*, pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral (*e.g.*, topical application) or enteral (*e.g.*, oral) which do not deleteriously react with the active compounds.

Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions, alcohols, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatine, carbohydrates such as lactose, amylose, or starch, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, pentaerythritol fatty acid esters, hydroxy methylcellulose, polyvinyl pyrrolidone, merely to name a few. The pharmaceutical

preparations can be sterilized and if desired mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifier, salts for influencing osmotic pressure, buffers, coloring, flavoring, and/or aromatic substances and the like which do no deleteriously react with the active compounds. They can also be
5 combined where desired with other agents, *e.g.* vitamins.

Additionally, the inhibitor may be introduced in a polymer gel for controlled release. Methods of producing such gels are set forth in U.S. Patent No. 5,567,435 to Hubbel *et al.*, herein incorporated by reference. Methods of injecting polymers are set forth in U.S. Patent No. 5,567,612 to Vacanti *et al.*, hereby incorporated by reference.
10 Moreover, in cases where the inhibitor chosen is a peptide, methods for incorporating peptides into polymers is described in U.S. Patent No. 5,482,996 to Russell *et al.*, herein incorporated by reference.

For enteral application, particularly suitable are tablets, liquids, drops, suppositories, or capsules. A syrup, elixir, or the like can be used wherein a
15 sweetened vehicle is employed. Sustained or directed release compositions can be formulated, *e.g.*, liposomes or those wherein the active compound is protected with differentially degradable coating, *e.g.*, by microencapsulation, multiple coatings, *etc.*

In this manner, the present invention may be introduced into a subject in polymeric microspheres for the controlled release of the compound. Methods of
20 producing microspheres from polymer can be found in U.S. Patent No. 5,601,844 to Kagayama *et al.* and U.S. Patent Nos. 5,529,914 and 5,573,934 to Hubbel *et al.*, herein incorporated by reference.

For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants
25 including suppositories. Nebulizers and inhalation aerosols may also be used. Ampules are in convenient unit dosages. It is also possible to freeze-dry the new compounds and use the lypophilizates obtained, for example, for the preparation of products for injection.

For other parenteral applications, such as topical applications and non-sprayable forms, viscous to semi-solid or solid forms comprising a carrier compatible with topical application and having a dynamic viscosity preferably greater than water. Suitable formulations include but are not limited to transdermal patches, solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, aerosols, *etc.*, which are, if desired, sterilized or mixed with auxiliary agents, *e.g.*, preservatives, stabilizers, wetting agents, buffers, or salts for influencing osmotic pressure, *etc.*

Also suitable for topical application are sprayable aerosol preparations wherein the inhibitor, preferably in combination with a solid or liquid inert carrier material, is packaged in a squeeze bottle or in admixture with pressurized volatile, normally gaseous propellant, *e.g.*, a freon. The application of these embodiments can be to the skin or mucous membrane or in the interior of the body and can be oral, peroral, enteral, pulmonary, rectal, nasal, vaginal, lingual, intervenous, intraarterial, intracardial, intramuscular, intraperitoneal, intracutaneous, subcutaneous. The parenteral preparations are preferably sterile or sterilized products.

In this manner, U.S. Patent No. 4,895,727 to Allen, herein incorporated by reference, describes a method of inducing a reservoir effect in skin and mucous membranes so as to enhance penetration and retention and reduce transdermal flux of topically applied therapeutic and cosmetic pharmacologically active agents. U.S. Patent No. 4,557,934 to Cooper, herein incorporated by reference, describes topical pharmaceutical compositions containing a pharmaceutically-active agent and the penetration enhancing agent, 1-dodecylazacycloheptan-2-one. This composition provides marked transepidermal and percutaneous delivery of the selected pharmaceutically-active agent.

Suppositories containing inhibitor can be created using a suitable oleaginous or water-soluble base. The oleaginous class includes cocoa butter and fats with similar properties: the water-soluble class includes polyethylene glycols.

Other medicaments containing inhibitor can be produced in a known manner, whereby the known and customary pharmaceutical adjuvants as well as other customary carrier and diluting agents can be used. Examples include, but are not

limited to, gelatins, natural sugars such as sucrose or lactose, lecithin, pectin, starch
 (for example cornstarch), alginic acid, tylose, talc, lycopodium, silica (for example
 colloidal silica), glucose, cellulose, cellulose derivatives for example, cellulose ethers
 in which the cellulose hydroxyl group are partially etherified with lower aliphatic
 5 alcohols and/or lower saturated oxyalcohols, for example, methyl hydroxypropyl
 cellulose, methyl cellulose, cellulose phthalate, stearates, *e.g.*, methylstearate and
 glyceryl stearate, magnesium and calcium salts of fatty acids with 12 to 22 carbon
 atoms, especially saturated acids (for example, calcium stearate, calcium laurate,
 magnesium oleate, calcium palmitate, calcium behenate and magnesium stearate),
 10 emulsifiers, oils and fats, especially of plant origin (for example, peanut oil, castor oil,
 olive oil, sesame oil, cottonseed oil, corn oil, wheat germ oil, sunflower seed oil,
 cod-liver oil), mono, di, and triglycerides of saturated fatty acids ($C_{12}H_{24}O_2$ to
 $C_{18}H_{36}O_2$ and their mixtures), *e.g.* glyceryl monostearate, glyceryl distearate, glyceryl
 tristearate, glyceryl trilaurate), pharmaceutically compatible mono- or polyvalent
 15 alcohols and polyglycols such as glycerine, mannitol, sorbitol, pentaerythritol, ethyl
 alcohol, diethylene glycol, triethylene glycol, ethylene glycol, propylene glycol,
 dipropylene glycol, polyethylene glycol 400, and other polyethylene glycols, as well as
 derivatives of such alcohols and polyglycols, esters of saturated and unsaturated fatty
 acids (2 to 22 carbon atoms, especially 10 to 18 carbon atoms), with
 20 monohydric aliphatic alcohols (1 to 20 carbon atom alkanols), or polyhydric alcohols
 such as glycols, glycerine, diethylene glycol, pentaerythritol, sorbitol, mannitol, ethyl
 alcohol, butyl alcohol, octadecyl alcohol, *etc.*, *e.g.* glyceryl stearate, glyceryl palmitate,
 glycol distearate, glycol dilaurate, glycol diacetate, monoacetin, triacetin, glyceryl
 oleate, ethylene glycol stearate; such esters of polyvalent alcohols can in a given case
 25 be etherified, benzyl benzoate, dioxolane, glycerine formal, tetrahydrofurfuryl alcohol,
 polyglycol ethers with 1 to 12 carbon atom alcohols, dimethyl acetamide, lactamide,
 lactates, *e.g.*, ethyl lactate, ethyl carbonate, silicones (especially middle viscosity
 dimethyl polysiloxane).

Other adjuvants can also be substances which bring about decomposition (so-called explosives) such as: cross-linked polyvinyl pyrrolidone, sodium carboxy methyl starch, sodium carboxy methyl cellulose or microcrystalline cellulose. Likewise, known coating agents such as *e.g.* polyacrylates, cellulose ethers and the like can be used.

For the production of solutions, there can be used water of physiologically compatible organic solvents, as for example, ethanol, 1,2-propylene glycol, polyglycols, *e.g.*, diethylene glycol, triethylene glycol and dipropylene glycol and their derivatives dimethyl sulfoxide, fatty alcohols, *e.g.*, stearyl alcohol, cetyl alcohol, lauryl alcohol and oleyl alcohol, triglycerides, *e.g.* glyceryl oleate glyceryl stearate, glyceryl palmitate, and glyceryl acetate, partial esters of glycerine, *e.g.*, glyceryl monostearate, glyceryl distearate, glyceryl monopalmitate, paraffins, and the like.

For injectable solutions or suspensions, non-toxic parenterally compatible diluting agents or solvents can be used, for example: Water, 1,3 butane diol, ethanol, 1,2-propylene glycol, polyglycols in a mixture with water, Ringer's solution, isotonic solution of sodium chloride or also hardened oils including synthetic mono or diglycerides or fatty acids like oleic acid.

Known and customary solution assistants or emulsifiers can be used in the production of the preparations. The following are examples of solution assistants and emulsifiers which can be used: Polyvinylpyrrolidone, sorbitan fatty acid esters such as sorbitan trioleate, phosphatides such as lecithin, acacia, tragacath, polyoxyethylated sorbitan monooleate and other ethoxylated fatty acid esters of sorbitan, polyoxyethylated fats, polyoxyethylated oleotriglycerides, linolized oleotriglycerides, polyethylene oxide condensation products of fatty alcohols, alkyl phenolene or fatty acids or also 1-methyl-3-(2-hydroxyethyl)imidazolidone-(2). The term polyoxyethylated means in this context that the substances in question contain polyoxyethylene chains whose polymerization is generally between 2 to 40 and especially between 10 to 20.

Such polyoxyethylated substances can be obtained, for example, by reacting compounds containing hydroxyl groups (*e.g.* mono or diglycerides or unsaturated compounds such as, *e.g.*, those containing the oleic acid residues) with ethylene oxide (*e.g.* 40 moles ethylene oxide per mole glyceride). Examples of oleotriglycerides are olive oil, peanut oil, castor oil, sesame oil, cotton seed oil and corn oil. [See also Fiedler, Lexicon der Hilfstoffe für Pharmazie, Kosmetik und angrenzende Gebiete [Lexicon of Adjuvants for Pharmacy, Cosmetics and Related Areas] pp. 191-195 (1971)].

Furthermore, there can be added preservatives stabilizers, buffers, for example, calcium hydrogen phosphate, colloidal aluminum hydroxide, taste correctives, antioxidants and complex formers (for example, ethylene diamine tetraacetic acid) and the like. In a given case for stabilization of the active molecule, the pH is adjusted to about 3 to 7 with physiologically compatible acids or buffers. Generally, there is preferred as neutral as possible to weak acid (to pH 5) pH value.

As antioxidants, there can be used, for example, sodiummeta bisulfite, ascorbic acid, gallic acid, alkyl gallates, *e.g.*, methyl gallate and ethyl gallate, butyl hydroxyanisole, nordihydroguararetic acid, tocopherols as well as tocopherol and synergists (materials which bind heavy metals by complex formation, for example, lecithin, ascorbic acid, phosphoric acid). The addition of synergists increases considerably the antioxidant activity of tocopherol. As preservatives, there can be used, for example, sorbic acid, p-hydroxybenzoic acid esters (for example, lower alkyl esters such as the methyl ester and the ethyl ester) benzoic acid, sodium benzoate, trichloroisobutyl alcohol, phenol, cresol, benzethonium chloride, and formalin derivatives.

Examples

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the following examples, the following abbreviations apply: IPF, inhibitory protein factor; BSA, bovine serum albumin; EDTA, (ethylenedinitrilo)-tetraacetic acid; GABA, γ -aminobutyric acid; HEPES, 4-(2 hydroxyethyl)-1-piperazine-ethanesulfonic acid; PAE, polyethyleneimine; PMSF, phenylmethyl sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; V-type H⁺-ATPase, vacuolar-type proton translocating ATPase.

In the following examples, the materials utilized were obtained as follows: Polyethyleneimine (PAE)-1000 anion-exchanger was purchased from Amicon Corporation. Hydroxylapatite HPT was from Bio-Rad. Superdex S200 26/60 and Mono Q 5/5 columns were purchased from Pharmacia. [³H]Glutamate (50 Ci/mmol) was purchased from Amersham Corporation. CytoScint ES scintillation fluid was from ICN. Whatman GFC filters were purchased from VWR. Ammonium sulfate was from Mallinckrodt. Protein was quantified with the Coomassie Protein Assay Kit from Pierce, using bovine serum albumin as standard. All other chemicals and chromatography media were purchased from Sigma.

Example 1: Purification and Use of IPF

In this example, the purification and use of an inhibitor of synaptic glutamate uptake is described. The example is divided into three sections: A) preparation of the synaptic vesicles and synaptosomes, B) purification of inhibitory protein factor (IPF), and C) inhibition of glutamate uptake by synaptic vesicles with IPF. All of the following procedures were performed on ice or in a 4 °C cold-room unless otherwise noted.

A. Preparation of Synaptic Vesicles and Synaptosomes

Bovine synaptic vesicles were prepared by a slight modification of the procedure described by Tabb *et al.* [(1992) *J. Biol. Chem.* 267, 15412-15418]. Briefly, bovine brains were obtained from the local slaughterhouse. On ice, the meninges were removed from each brain, the cerebral cortex dissected, and excess white matter removed. About 300 grams of cortex were briefly blended in 600 ml of

0.32 M sucrose, 1 mM NaHCO₃, 1 mM magnesium acetate, 0.5 mM calcium acetate, 0.2 mM phenylmethylsulfonyl fluoride (solution A) in a Waring blender with 3 x 7 second bursts; solution A was used in all steps unless noted otherwise.

5 The homogenate was diluted to two liters in solution A and was rehomogenized in a 300-ml tight fitting Teflon-glass homogenizer (Kontes, Vineland, NJ) with two strokes, at 1900 rpm. Each batch was diluted to 3 liters and was centrifuged in a Sorvall GSA rotor at 2500 rpm for 10 minutes. The supernatant (S1) was saved, and the pellets (P1) from various batches were pooled, diluted to 4 liters, rehomogenized and recentrifuged at 2500 rpm for ten minutes. This supernatant (S1) was pooled with
10 the previous S1 and was centrifuged in a GSA rotor at 13,000 rpm for 15 minutes. This pellet was saved, and the supernatant was discarded.

The P2 was resuspended in solution A to 1.6 liters. This suspension was diluted with an equal volume of 1.28 M sucrose, to make the final concentration of sucrose 0.8 M, and was then centrifuged at 13,000 rpm in a GSA rotor for 45 minutes.
15 The floating myelin bands and supernatant were aspirated, and the pellets (synaptosomes) were saved. The synaptosomes were resuspended in two liters of ice-cold lysing buffer (6 mM Tris-maleate, pH 8.1), diluted to 8 liters lysing buffer, mechanically stirred at 4°C for 45 minutes and were then centrifuged at 19,000 rpm in a Sorvall SS-34 rotor for 15 minutes. The supernatant was then concentrated from 8
20 liters to 800 ml in an Amicon spiral ultraconcentrator, equipped with an S1Y30 cartridge (30,000 molecular weight cutoff). The retentate was then centrifuged at 43,000 rpm in a Beckman 45Ti rotor ultracentrifuge rotor for 70 minutes.

The pellets (crude synaptic vesicles) were resuspended in 20 ml of lysing buffer and were layered over six discontinuous sucrose gradients (12 ml 0.4 M, 6 ml 0.6 M),
25 and were then centrifuged at 35,000 rpm in a Beckman Type 45Ti rotor for two hours. The lysing buffer and 0.4 M sucrose layers (but not the 0.4 M, 0.6 M sucrose interface; it contains plasma membrane contaminants) were removed, diluted with lysing buffer and were centrifuged at 47,000 rpm in a Beckman Ti50 rotor for 60

minutes. The pellets were saved and were either resuspended in solution B (0.32 M sucrose, 1 mM NaHCO₃, 1 mM dithiothreitol) at about 5 mg/ml and stored in liquid nitrogen and were stable for at least one month.

Bovine synaptosomes used in Figure 5 (see description below) were collected during the preparation of synaptic vesicles [Tabb *et al.* (1992) *J. Biol. Chem.* 267, 15412-15418]. Synaptosomes were resuspended in normal Krebs-Ringers (0.15 M NaCl, 6.2 mM KCl, 1.2 mM Na₂HPO₄, 1.2 mM MgSO₄, 10 mM glucose, 20 mM Tris-HEPES, pH 7.4) or low Na⁺ Krebs-Ringers (substituting 0.15 M choline chloride for NaCl) prior to assay.

B. Purification of IPF

25 calf brains were obtained fresh from a local slaughterhouse, and the cerebellum, brain stem, and excess white matter removed to yield 5,500-6,000 grams of cerebral tissue. A Waring blender was used to mince 300 grams of cortex at a time in 800 ml of 1 mM PMSF, 1 mM EDTA, 5 mM 2-mercaptoethanol, 6 mM Tris-HCl, pH 8.3 (*lysing buffer*). The blended material was then diluted to ~40 liters in *lysing buffer* and homogenized to smoothness by passing through a large, continuous-flow homogenizer. The entire suspension was then centrifuged at 13,000 rpm (27,300 g_{max}) for 15 min in a Sorvall GSA rotor. The resulting supernatant (~ 25 liters) was concentrated to 10 liters in an Amicon spiral ultra-concentrator equipped with a S1Y30 cartridge (30,000 molecular weight cut-off) prior to further fractionation.

The inhibitory protein factor was purified to apparent homogeneity as follows:

Step 1: Ammonium sulfate precipitation - The crude cellular extract (~ 10 liters) was adjusted to 45% saturation with ammonium sulfate and incubated for 30 min. The precipitate was collected by centrifugation for 15 min at 13,000 rpm (27,300 g_{max}), resuspended to 2.5 liters in *lysing buffer* and dialyzed overnight against 55 liters of same. The dialyzed sample was then clarified by centrifuging for 70 min at 45,000

rpm (235,400 g_{\max}) in a Beckman Type 45Ti rotor. The resulting supernatant (2.6 liters, 24,000 mg protein) was generally stored overnight at -20 °C prior to anion-exchange chromatography.

5 *Step 2: Anion-exchange chromatography* - One half of the 45% ammonium sulfate precipitate at a time (~ 1.3 liters) was loaded onto a PAE-1000 column (7.5 cm x 32 cm) equilibrated with *lysing buffer*, at a flow-rate of 50 ml/min. After collection of the flow-through fraction, bound protein was eluted with 2 liters each of 0.2, 0.5, and 1.0 M NaCl dissolved in the column buffer. The 0.5 M NaCl eluate (1.5 liters) was dialyzed overnight against 58 liters of a solution containing 1 mM $MgCl_2$, 0.2 mM PMSF, and 10 mM Tris-maleate, pH 8.0 (*HAP column buffer*). The 0.5 M NaCl eluate was usually fractionated on hydroxylapatite immediately following dialysis.

10 *Step 3: Hydroxylapatite column chromatography* - This step was typically run twice, with each run utilizing one of the two 0.5 M NaCl eluates obtained from the PAE-1000 column. The dialyzed PAE 0.5 M eluate (1.6 liters, 3,500 mg protein) was applied to a hydroxylapatite column (7.5 cm x 9 cm) equilibrated with *HAP column buffer* at a flow-rate of 20 ml/min. Bound protein was eluted with increasing steps of potassium phosphate (0.01, 0.05, 0.1, and 1 M) dissolved in *HAP column buffer*. The 0.05 M eluate was collected and later combined with the same fraction obtained from the second column run. The combined HAP 0.05 M eluates were adjusted to 80% saturation with ammonium sulfate, and the precipitates collected and resuspended to 200 ml in a solution containing 1 mM EDTA, 0.2 mM PMSF, and 10 mM Tris-maleate, pH 7.0 (*yellow column buffer*). This was dialyzed overnight against two 18-liter changes of the same.

20 *Step 4: Reactive Yellow-86 chromatography* - The dialyzed 0.05 M phosphate eluate from the hydroxylapatite column (250 ml, 1,800 mg protein) was loaded onto a Reactive Yellow-86 agarose column (4.5 cm x 22 cm) equilibrated with *yellow column buffer*. Approximately 95% of loaded protein does not bind to the column and is collected in the flow-through. Bound protein is eluted with successive steps of 0.06, 0.3, and 1 M NaCl dissolved in column buffer, at a flow-rate of 14 ml/min. The 0.3

M NaCl eluate (250 ml, 60 mg protein) was adjusted to 80% saturation with ammonium sulfate and the precipitate collected as previously described. The precipitate was resuspended to 10 ml in *yellow column buffer* and dialyzed overnight against two 5-liter changes of the same.

5 *Step 5: Gel filtration on Superdex S200* - The dialyzed Yellow-86 0.3 M NaCl eluate (10 ml, 18-20 mg protein) was applied to a Superdex S-200 26/60 column (62 cm x 65 cm) equilibrated with a solution containing 75 mM KCl, 1 mM EDTA, 0.2 mM PMSF, and 10 mM Tris-maleate, pH 7.0. The column was run at 1 ml/min, and 6 ml fractions collected (60 total). Typically, fractions 23-30 were pooled, and an
10 80% ammonium sulfate precipitate collected as previously described. Precipitate was resuspended in 2 ml of a solution containing 1 mM EDTA, 0.2 mM PMSF, and 10 mM Tris-maleate, pH 7.0, and dialyzed against 4 liters of the same for 4 hours just prior to sucrose gradient centrifugation.

 The estimated Stokes radius was determined by utilizing plots of $(-\log K_{av})^{1/2}$ vs.
15 the Stokes radius of standard proteins (ferritin, 79 Å; BSA, 35 Å; and myoglobin, 17 Å) according to [Siegel and Monty (1966) *Biochim. Biophys. Acta* 112, 346-362].

Step 6: Sucrose density gradient centrifugation - The dialyzed, ammonium sulfate precipitate from the Superdex S-200 column (2.5 ml, 20-25 mg protein) was layered onto two 36 ml 5-20% sucrose gradients developed in a solution containing 50
20 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, and 10 mM HEPES, pH 7.4. Gradients were centrifuged for 43 hr at 28,000 rpm ($140,000 \times g_{max}$) in a Beckman SW28 rotor. A gradient containing catalase (11.3 S), BSA (4.3 S), and myoglobin (2.1 S) was also run in parallel in order to determine the sedimentation coefficients in Table II below [Martin and Ames (1960) *J. Biol. Chem.* 236, 1372-1379]. Typically, thirty-six 1-ml
25 fractions were collected from each gradient by puncturing the tube bottoms.

Step 7: HPLC anion-exchange chromatography - Peak inhibitory fractions from the sucrose gradients (typically fractions 22-26, ~2 mg protein) were pooled and applied to a Pharmacia Mono Q 5/5 anion-exchange column equilibrated with a solution containing 75 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, and 20 mM Tris-HCl,

pH 7.6. Bound protein was eluted with a linear NaCl gradient (75-538 mM) developed over 60 min at a flow-rate of 1 ml/min. Fractions were collected (60 x 1 ml), and 10- μ l aliquots were assayed for inhibitory activity. Controls were performed by assaying aliquots from an identical gradient run in the absence of added protein.

5 Typical results of the procedure used to purify IPF α and IPF β are shown in Table I and in Figures 2 and 3 (described below). Hydroxylapatite chromatography at pH 8.0 in the presence of Mg^{2+} proved to be a critical step during purification. Under these conditions, about 50% of the inhibitory activity was found in the 0.05 M phosphate eluate and resolved from a second peak of inhibitory activity present in the
10 0.1 M eluate. This accounted for the 50% loss of total inhibitory activity seen at this step. The activity in 0.1 M eluate correlated with a 73 kDa protein which may represent a proteolytic digestion product of IPF (EDÖ and TU, unpublished observation).

 The dye Reactive Yellow 86 was found to have a particularly selective affinity
15 for IPF. The 0.3 M NaCl eluate from this column provided the first glimpse of the IPF $\alpha\beta\gamma$ triplet against the protein background (Figure 2, lane 6, described below). Greater than 90% of the loaded protein did not bind to this column and, although the column effluent contained the majority of the inhibitory activity, no increase in specific activity was achieved.

20 Sucrose gradient centrifugation proved effective in the purification of IPF because of the anomalous sedimentation behavior of the IPF triplet. IPF α , β , and γ all migrated identically in 5-20% sucrose gradients, with an apparent sedimentation coefficient of 4.3 S. This step usually yielded at least one fraction that was essentially a purified preparation of IPF $\alpha\beta\gamma$.

25 These steps and their resulting levels of purification are summarized in Table I, indicating a 1,160-fold to 1,280-fold purification for IPF α and IPF β respectively.

Table I: Purification of IPF from calf brain cytosol.

Fraction	Total Protein (mg)	IC ₅₀ (mg/.12ml)	Total Activity (U)	Yield (%)	Specific Activity (U/mg)	Purificat. (-fold)
Cytosol	69,960	0.50	139,920	100	2	1
45% AS	26,920	0.25	107,680	77	4	2
PAE 0.5M	6,952	0.081	85,827	61	12	6
HAP 0.05M	1,824	0.040	45,600	33	25	13
Yellow 0.3M	58	0.011	5,273	3.8	91	46
Superdex Peak	23	0.0050	4,600	3.3	200	100
Sucrose Peak	2.5	0.0011	2,273	1.6	909	455
Mono Q IPF α	0.25	0.00043	581	0.42	2,326	1,160
Mono Q IPF β	0.25	0.00039	641	0.46	2,564	1,280

Figure 2 displays SDS-PAGE profiles of the products in the steps of the purification procedure. The starting material and fractions containing the peak inhibitory activity from the various purification steps were dissociated by boiling for 2 min in the presence of 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 63 mM Tris-HCl, pH 6.8, and subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel. Gel staining was with Coomassie Brilliant Blue. Lane 1, 40 μ g calf-brain homogenate; lane 2, 40 μ g crude cytosol; lane 3, 40 μ g 45% ammonium sulfate precipitate; lane 4,

40 μ g PAE 0.5 M NaCl eluate; lane 5, 40 μ g HAP 0.05 M phosphate eluate; lane 6, 40 μ g Yellow 86 0.3 M NaCl eluate; lane 7, 20 μ g gel filtration peak; lane 8, 5 μ g sucrose gradient peak; lane 9, 1.5 μ g Mono Q-purified IPF α ; lane 10, 1.5 μ g Mono Q-purified IPF β .

5 Figure 3 compares the SDS-PAGE profile, (upper panel), of peak inhibitory fractions as eluted from Mono Q HPLC, (lower panel). Peak inhibitory fractions from two 5-20% sucrose density gradients (12 ml, 2 mg protein) were applied to a Mono Q 5/5 anion-exchange column equilibrated in 75 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, 20 mM Tris-HCl, pH 7.6. Bound protein was eluted in sixty 1-ml fractions by
10 a linear NaCl gradient (75-538 mM) developed over 60 min. Aliquots of 10 μ l were assayed for inhibitory activity (upper panel) and for composition by SDS-PAGE (lower panel). All of the inhibitory activity eluted between gradient fractions 24 and 35. Samples for SDS-PAGE were dissociated and electrophoresed. Gel staining was with Coomassie Brilliant Blue.

15 Figure 3 shows that high resolution anion-exchange chromatography can partially resolve IPF $\alpha\beta\gamma$ into individual components based on assumed differences in net negative charge. It can also be concluded from this figure that both IPF α and IPF β possess inhibitory activity. Fraction 30 (see Figure 2) usually contained a mixture of IPF α,β and γ , reminiscent of the starting material, while fraction 29 usually contained
20 roughly equal amounts of IPF β and γ . Fraction 28 contained very pure IPF β (135 kDa), and later fractions contained pure IPF α (138 kD).

C. Inhibition of Glutamate Uptake By Synaptic Vesicles

The uptake of [3 H]-glutamate into synaptic vesicles was assayed using a modification of the filtration procedure described in [Kish and Ueda (1989) *Meth. Enzymol.* 174, 9-25]. Briefly, synaptic vesicles (30-50 μ g-protein) were suspended in
25 120 μ l of an incubation medium consisting of 0.23 M sucrose, 4 mM KCl, 4 mM MgSO₄, 2 mM aspartate, 10 mM methionine sulfoximine, 1 mM spermine, \pm 2 mM ATP, \pm IPF sample 10 mM HEPES, pH 7.4, and 50 μ M [3 H]glutamate (specific activity, 0.017 Ci/mmol). Glutamate uptake was initiated by transferring the mixtures

from ice to a 30 °C water bath, and the uptake reaction was allowed to proceed for 10 min. Baseline ATP-dependent glutamate uptake activity was calculated as the glutamate taken up in the presence of ATP minus that taken up in the absence of ATP. Throughout this work, glutamate uptake activity refers to the ATP-dependent portion, which was typically greater than 90% of the total. One unit (U) of the inhibitory protein factor was defined as the amount of protein required to inhibit 50% of ATP-dependent glutamate uptake over a period of 10 min at 30 °C.

Figure 4 shows that IPF α is a potent inhibitor of ATP-dependent glutamate uptake in synaptic vesicles. Purified bovine synaptic vesicles (50 μ g of protein) were suspended in the glutamate uptake assay medium in the presence of varying amounts of fraction 31 from the Mono Q column (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.65, 0.76, 0.86, 0.97, 1.1, 1.3, and 1.6 μ g protein). Using a molecular weight of 138,000 for IPF α , these amounts were converted to 26 nmol IPF α /liter. Uptake was allowed to proceed for 10 min at 30 °C. Values for % of control were calculated relative to the ATP-dependent uptake in samples containing equivalent amounts of fraction 31 from a Mono Q gradient run in the absence of loaded protein.

A similar dose-response curve for IPF β was also generated and indicated an IC_{50} of 24 nM (data not shown). At approximately 100 nM, both IPF α and β inhibited ATP-dependent glutamate uptake by 90%. However, neither IPF α nor IPF β had any effect on the ATP-independent component of glutamate uptake even at concentrations up to 100 nM (data not shown).

In order to investigate the specificity of the inhibitory effect produced by IPF, its effect on uptake in two other well characterized systems was examined: the Na^+ -dependent glutamate uptake system in the synaptosomal plasma membrane and the ATP-dependent, reserpine-sensitive catecholamine uptake system in chromaffin vesicles from the adrenal medulla.

Figure 5 depicts the results of this examination. Bovine synaptic vesicles (50 μ g of protein) were suspended in glutamate uptake assay medium. Bovine synaptosomes (35 μ g protein) were suspended in 0.12 ml of Krebs-Ringer solution containing 1 mM spermine and 1 μ M [3H]glutamate (1.67 Ci/mmol) in the presence of

Na⁺ (150 mM) or choline (150 mM). Bovine chromaffin vesicles (45 μg protein) were suspended in 0.12 ml of a solution containing 0.3 M sucrose, 1 mM spermine, 10 mM MgSO₄, 5 mM ATP, 10 mM HEPES, pH 7.0 and 50 μM [³H]norepinephrine (0.017 Ci/mmol) with or without 1 μM reserpine. Each membrane mixture also contained either 0, 26, or 100 nM IPF α. Glutamate uptake was allowed to proceed for 10 min at 30 °C and 30 min at 37 °C for norepinephrine uptake. Reaction was terminated by filtration as described in Methods. Values for % of control were calculated relative to the specific uptake activity obtained in the absence of IPF α. Uptake into synaptic vesicles is represented by ATP-dependent uptake, that into synaptosomes by Na⁺-dependent uptake, and that into chromaffin vesicles by reserpine-sensitive uptake.

Results in Figure 5 indicate that IPF α exhibited no inhibitory effect on Na⁺-dependent glutamate uptake into bovine synaptosomes. Moreover, IPF α had only a minimal effect (~ 18% inhibition) on norepinephrine uptake into bovine chromaffin vesicle ghosts at 100 nM, a concentration that inhibited vesicular glutamate uptake by 90%.

The precise mechanism by which IPF leads to inhibition of vesicular glutamate uptake remains to be determined. Since transport of glutamate into synaptic vesicles is a coupled process, the possibilities for inhibiting such a system are multiplied by the number of potential coupling sites. Indirect modes of inhibition would include inhibiting the activity of the V-type H⁺-ATPase, increasing the passive permeability of the vesicle membrane to protons, or causing a generalized increase in membrane permeability (a detergent-like effect). The results in Figure 5 indicate that these possibilities are unlikely. If IPF were inhibiting the action of the V-type H⁺-ATPase (either ATP hydrolysis or proton pumping), it would be expected that norepinephrine transport into chromaffin vesicles would also be inhibited. Similarly, any inhibitor which has a generalized protonophore activity would also lead to decreased uptake into chromaffin vesicles. Figure 5 shows that norepinephrine transport is hardly affected by IPF concentrations up to 100 nM. Finally, the generalized increase in membrane

permeability characteristically caused by detergents and other amphiphilic molecules should have an effect on glutamate storage in synaptosomes as well as on norepinephrine uptake. This was not observed.

Even though it has not been possible thus far to isolate purified GABAergic vesicles, preliminary experiments with mixed vesicle preparations (containing both glutamate and GABA uptake activities) have shown IPF to be just as potent an inhibitor of vesicular GABA uptake (data not shown).

Example 2: Characterization of IPF

Some of the physicochemical properties of IPF are summarized in Table II.

Table II:

Property	IPF α	IPF β	IPF γ
Relative Molecular Wt	138,000	135,000	132,000
Molecular Wt	103,500	-	-
Stokes Radius (angstroms)	60	60	60
Sedimentation Coefficient	4.3	4.3	4.3
Partial Specific volume (ml/gm)	0.717	-	-
Frictional ratio (f/f_0)	1.67	-	-
Axial ratio	12	-	-
IC ₅₀ (nM)	26	24	-

IPF α , β and γ share highly similar physicochemical properties. The relative apparent molecular weights determined by SDS-PAGE were 138, 135, and 132 x 10³ for IPF α , β and γ , respectively. Their Stokes radii and sedimentation coefficients were indistinguishable from each other, being 60Å and 4.3S, respectively. The partial specific volume of IPF α was calculated to be 0.717 ml/g from amino acid composition. Using these values, an approximate molecular weight of IPF α in the native form was calculated to be 103,000, according to the equation $M = \frac{6\pi\eta N a s}{(1-\bar{v}\rho)}$, where N = Avogadro's number, η = viscosity of water at 20 °C, a = Stokes radius, s = sedimentation coefficient at 20 °C, \bar{v} = partial specific volume, and ρ = density of water at 20 °C [Siegel and Monty (1966) Biochim. Biophys. Acta 112, 346-362]. This molecular weight is significantly smaller than that determined by SDS-PAGE. The excessively high Stokes radius and low sedimentation coefficient values for a globular protein indicate that IPF has an elongated shape. This is in agreement with the rather high axial ratio of 12 estimated from the calculated frictional coefficient ratio of 1.67.

Calculations utilizing the Stokes radius, sedimentation coefficient, and partial specific volume for IPF α indicate a native molecular mass of 103 kDa. This is at odds with the apparent molecular weight of 138,000 determined by SDS-PAGE. Additionally, the unexpectedly low sedimentation coefficient of 4.3 S is not consistent with the large Stokes radius (60 Å). These data collectively indicate that IPF α is a protein with a markedly elongated structure. This hypothesis is further supported by the large frictional coefficient and axial ratio (see Table II) determined for IPF α and by the relationship to α fodrin, itself a linear protein. How this rather eccentric structural characteristic might contribute to the ability to inhibit glutamate uptake is not known. The differences in charge between IPF α and IPF β , which render them separable by ion-exchange HPLC (see Figure 3), apparently have little effect on inhibitory activity, as the two proteins share similar IC₅₀ values.

Figure 6A gives the results of partial sequencing of IPF α . N-terminal sequencing revealed that amino acids 1-20 of IPF α , β and γ are identical with amino acids 26-45 of human α fodrin. Four further peptides (amino acids 393-415, 621-636,

965-974, and 1086-1095) generated by partial digestion of IPF α confirmed the relationship to α fodrin. Amino acid sequences determined for IPF α are shown in boldface in Figure 6A within the initial 1200 amino acid residues of human α fodrin as determined by Moon and McMahon [(1990) *J. Biol. Chem.* 265, 4427-4433]. The 20-mer beginning with Tyr²⁶ represents the N-terminus of IPF α . The four internal sequences were determined by sequencing peptides produced by proteolytic digestion of IPF α . The highlighted bond between Tyr¹¹⁷⁶ and Gly¹¹⁷⁷ represents the cleavage site for calpain [Harris *et al.* (1988) *J. Biol. Chem.* 263, 15754-15761]. Figure 6B shows the predicted sequence of IPF based upon sequencing of the N-terminus and the cleavage site for calpain as the C-terminus. Based upon this sequence, IPF α is estimated to have a true molecular weight of about 133 kD.

Despite this relationship, fodrin purified from whole-brain, according to the method described by Cheney *et al.* [(1986) *Meth. Enzymol.* 134, 42-54], had no effect on glutamate uptake at concentrations up to 1 μ M (data not shown).

Example 3: Treatment of IPF with Trypsin

IPF partially isolated as described in Example 1 was further treated by incubation at 30°C for five minutes in the presence of trypsin (0.1 μ g). Figure 7 shows the effect of trypsin exposure on IPF. Lane A shows the SDS-PAGE profile of the partially purified IPF composition without trypsin treatment. Lane B shows the SDS-PAGE profile of the partially purified IPF composition with trypsin treatment. It is clear that the 138 kD IPF band is not present, demonstrating the digestion of IPF by trypsin. Lane C shows the SDS-PAGE profile of the partially purified IPF composition obtained in the presence of trypsin and pancreatic trypsin inhibitor. Thus, it is clear that treatment of IPF with trypsin induces digestion of the IPF proteins.

The impact of trypsin digestion on glutamate uptake inhibition in synaptic vesicles was then determined. Figure 8 depicts these results. Lane A depicts control glutamate uptake without additives. Lane B depicts glutamate uptake by synaptic vesicles in the presence of trypsin and pancreatic trypsin inhibitor. Lane C depicts glutamate uptake by synaptic vesicles in the presence of partially purified IPF. Lane

D depicts glutamate uptake by synaptic vesicles in the presence of partially purified IPF and trypsin. Lane E depicts glutamate uptake by synaptic vesicles in the presence of partially purified IPF, trypsin and pancreatic trypsin inhibitor. While Lanes A and B show no significant glutamate uptake inhibition, both the unfragmented IPF in Lanes C and E and the trypsin fragmented IPF of Lane D showed glutamate uptake inhibition. Thus, it is clear that one or more products resulting from trypsin digestion of IPF inhibit glutamate uptake by synaptic vesicles.

Example 4: Animal Model for *In vivo* Drug Screening

As described above, the present invention contemplates the use of synaptic vesicle glutamate uptake inhibitors for the treatment of neurosynaptic disorders. Screening of glutamate uptake inhibitors for *in vivo* efficacy can be performed in the context of animal models. One animal model is the Epileptic (EL) mouse.

The seizures in EL mice are inherited as a multifactorial trait and are considered model for human complex partial seizures with secondary generalization [Rise *et al.* (1991) *Science* 253 669-73]. The seizures in EL mice occur spontaneously or can be induced by rhythmic vestibular stimulation [Kurokawa *et al.* (1966) *Prog. Brain Res.* 21A 112-30]. The seizures in EL mice generally begin at 80 to 100 days of age and are thought to originate in or near the parietal lobe and then spread quickly to the hippocampus and to other brain regions [Seyfried (1992) *Neurosciences* 18 (Suppl. 2) 9-20].

In this example, it is shown that synaptic vesicle glutamate uptake is increased in a brain region-specific manner in epileptic (EL) mice as compared to age matched non-epileptic control mice. This increase was observed at an age when EL mice express handling-induced seizures, but it was not seen in young EL mice prior to seizure onset. The findings could reflect an increase in glutamatergic synaptic vesicle number, with or without a change in the number of nerve terminals, or alternatively an increase in the number of nerve terminals without an increase in vesicles per terminal.

A combination of these mechanisms might also be involved. It is also possible that this increase is due to alterations in the synaptic vesicle such as regulation of transport by chloride or an internal volume change in the synaptic vesicle.

Synaptic vesicles isolated from various brain regions in EL mice prior to (46
5 days old) and after (approx. 400 days old) the onset of handling or spontaneous seizures are studied. The EL mice were compared to age-matched nonepileptic control mice (DDY and APB).

Synaptic vesicles were prepared from four brain regions: cerebrum (minus
10 hippocampus), hippocampus, cerebellum and brain stem. The cerebrum therefore contains the entire cerebral cortex, except for the hippocampus. Each preparation represents the synaptic vesicles from one mouse with the exception of the hippocampus, where each preparation represents 1-2 mice. Each tissue preparation represents different mice.

In brief, brain tissue was homogenized in solution A (0.32 M sucrose, 0.5 mM
15 calcium acetate, 1 mM magnesium acetate and 1 mM NaHCO_3). The homogenate was centrifuged at 10,000 rpm in a Sorvall SM-24 rotor for 20 minutes. The pellets were lysed for 45 minutes in 6 mM Tris-maleate (pH 8.1) and centrifuged at 19,000 rpm in a Sorvall SM-24 rotor for 15 minutes. The supernatant was centrifuged at 43,000 rpm in a Beckman Ti45 rotor for 70 minutes, and the crude synaptic vesicles were
20 resuspended in solution B (0.32 M sucrose, 1 mM dithiothreitol and 1 mM NaHCO_3). Synaptic vesicles were stored in liquid nitrogen until use, typically within one week. Synaptic vesicles prepared in this manner exhibited properties essentially indistinguishable from those observed with highly purified preparations.

Synaptic vesicle uptake activity was determined by the method described above.
25 In the old mice, glutamate uptake activity was significantly increased in the EL mice cerebellum (minus hippocampus) compared to the control DDY mice. No difference was observed between EL and control DDY mice in the other brain areas. In younger mice, no significant difference in ATP-dependent glutamate uptake activity was observed either in the cerebrum (minus hippocampus) or in the brain stem. These
30 results are set forth in Table III.

Table III: Vesicular Uptake in EL and Control Non-Epileptic Mice

Strain	Days Old	Cerebrum (pmol/mg protein)	Hippocamp (pmol/mg protein)	Cerebellum (pmol/mg protein)	Brain Stem (pmol/mg protein)
EL	411±10	1230±59	766±107	358±55	466±66
DDY	395±8	813±69	689±88	316±11	374±43
ABP	424±6	796±64	632±123	N.D.	N.D.
EL	46±0	941±61	N.D.	N.D.	872±119
DDY	46±0	906±84	N.D.	N.D.	1078±181

The data suggest that ATP-dependent glutamate uptake increases in response to seizures. No difference were found between epileptic and non-epileptic mice prior to the onset of seizures (46 days old). The ATP-dependent glutamate uptake was increased in the epileptic mice in a brain-region specific manner at an age when the EL mice had a long seizure history. The data therefore suggest that the increased synaptic vesicle glutamate uptake in EL mice is not the initial cause of seizure development, but either an effect of the seizures or the cause of continued seizures.

Previous studies of glutamate levels in EL mice were concerned with changes in the total levels in brain tissue, including pools for both metabolism and synaptic release. It was difficult to draw coherent conclusions from some of these studies, as the results were not always consistent. Since the neurotransmitter pool represents a small portion of the total tissue glutamate content, these changes may not directly reflect changes in the levels of glutamate to be released as a neurotransmitter. Recent findings suggest that enhanced aspartate release may be genetically associated with seizure susceptibility in EL mice.

While no differences were observed in other brain regions between epileptic and nonepileptic mice, it was observed that ATP-dependent glutamate uptake activity

in the brain stem was higher in the 46 day old mice compared to the 400 day old mice. It is likely that this relates to maturation effects. It is unlikely that this relates to seizure development, as the control strain demonstrated uptake activity indistinguishable from that observed in the epileptic strain, regardless of age.

5 **Example 5: Polyclonal Antibodies to IPF**

In this example, antibodies were raised that were demonstrated to be reactive with both intact fodrin and a fodrin fragment (i.e. IPF alpha). Moreover, following absorption of the antibody mixture on column of immobilized intact fodrin, antibodies reactive with intact fodrin were removed, leaving antibodies only reactive with the
10 fodrin fragment.

Polyclonal antibodies to a decapeptide having a sequence corresponding to the N-terminal sequence of IPF α (anti-IPF α) were made in rabbits. The total protein from 50 ug Cytosol were then separated on SDS-PAGE. One strip of the gel was stained with Coomassie Blue. The proteins in the remaining portion of the gel were
15 transferred to nitrocellulose, the nitrocellulose was temporarily stained to visualize the lanes, destained, and blocked. The blot was cut into strips and each strip incubated with the appropriate primary antibody. After washing in buffer, antibody binding was visualized using a goat anti-rabbit IgG conjugated to alkaline phosphatase from Bio-Rad.

20 The results of the immunoblotting are shown in Figure 9. Panel A is a 7.5% polyacrylamide gel and containing 50 micrograms of cytosol. In this gel, the sample was probed with a 1:2000 dilution of unabsorbed rabbit anti-decapeptide. The gel shows that the unabsorbed antibody reacts with both intact fodrin and IPF α .

25 The antibody used in Panel A was thereafter absorbed on a column containing purified fodrin coupled to sepharose 4B. Total protein from a 100 ug Cytosol preparation was run (along with IPF alpha) on a gel (6.0% polyacrylamide gel) and transferred to nitrocellulose. The immunoblot was probed with a 1:1000 dilution of antiserum. The results are shown in Panel B. The immunoblot shows binding of

antibody to IPF α but no antibody binding to intact fodrin. This suggests that the conformation of intact fodrin is such that at least a portion of the region represented by the decapeptide is sterically hidden.

5 Based upon the description and experimental materials presented above, it is clear that the present invention provides compositions and method for the treatment of neurosynaptic disorders in a subject.

CLAIMS

We claim:

1. A composition comprising a purified fragment of fodrin having glutamate uptake inhibition activity, said fragment having an N-terminus and a C-terminus.

5 2. The composition of Claim 1, wherein said N-terminus corresponds to Tyr²⁶ of fodrin.

3. The composition of Claim 1, wherein said purified fragment comprises a peptide having the amino acid sequence EAALTSEEVG within 150 amino acids of the C-terminus of the peptide.

10 4. The composition of Claim 1, wherein said purified fragment comprises IPF α .

5. The composition of Claim 1, comprising a purified fragment of IPF α .

6. The composition of Claim 1, wherein said purified fragment comprises IPF β .

7. The composition of Claim 1, wherein said purified fragment comprises IPF γ .

15 8. A composition comprising a purified peptide having glutamate uptake inhibition activity with an N-terminus sequence comprising the amino acids YHRFK.

9. The composition of Claim 8, wherein said purified peptide has an N-terminus comprising the amino acids YHRFKELSTL.

10. The composition of Claim 9, wherein said purified peptide has an N-terminus comprising the amino acids YHRFKELSTLRRQKLEDSYR.

11. A method of isolating a glutamate uptake inhibitor, comprising:

- a) providing an animal brain; and
- b) subjecting said animal brain to a purification procedure such that a purified fodrin fragment having glutamate uptake inhibition activity is produced.

12. The method of Claim 11, wherein said purified fragment comprises IPF α .

13. The method of Claim 11, wherein said purified fragment comprises IPF β .

14. The method of Claim 11, wherein said purified fragment comprises IPF γ .

15. A method for assessing overcoming synaptic vesicle glutamate uptake inhibition activity, comprising:

- a) providing:
 - i) synaptic vesicles,
 - ii) as purified fodrin fragment having glutamate uptake inhibition activity, and
 - iii) candidate compound; and
- b) combining said candidate compound with said synaptic vesicles and said fragment such that the effect of said candidate compound on glutamate uptake by said synaptic vesicles can be assessed.

16. The method of Claim 15, wherein said purified fragment comprises IPF α .

17. The method of Claim 15, wherein said purified fragment comprises IPF β .

18. The method of Claim 15, wherein said purified fragment comprises IPF γ .

19. A method for assessing overcoming synaptic vesicle glutamate uptake inhibition activity, comprising:

a) providing:

i) synaptic vesicles,

ii) a purified fragment of IPF having synaptic vesicle glutamate uptake inhibition activity, and

iii) candidate compound; and

b) combining said candidate compound with said synaptic vesicles and said purified fragment such that the effect of said candidate compound on said

fragment's effect on glutamate uptake by said synaptic vesicles can be assessed.

20. The method of Claim 19, wherein said fragment comprises a fragment of IPF α .

21. The method of Claim 19, wherein said fragment comprises a fragment of IPF β .

22. The method of Claim 19, wherein said fragment comprises a fragment of IPF γ .

ABSTRACT

Compositions and methods for treating neurosynaptic disorder in a subject are described. More specifically, compositions and methods for inhibiting glutamate uptake by synaptic vesicles in a subject are set forth. In one embodiment, the composition is inhibitory protein factor (IPF) and the subject is a human.

5

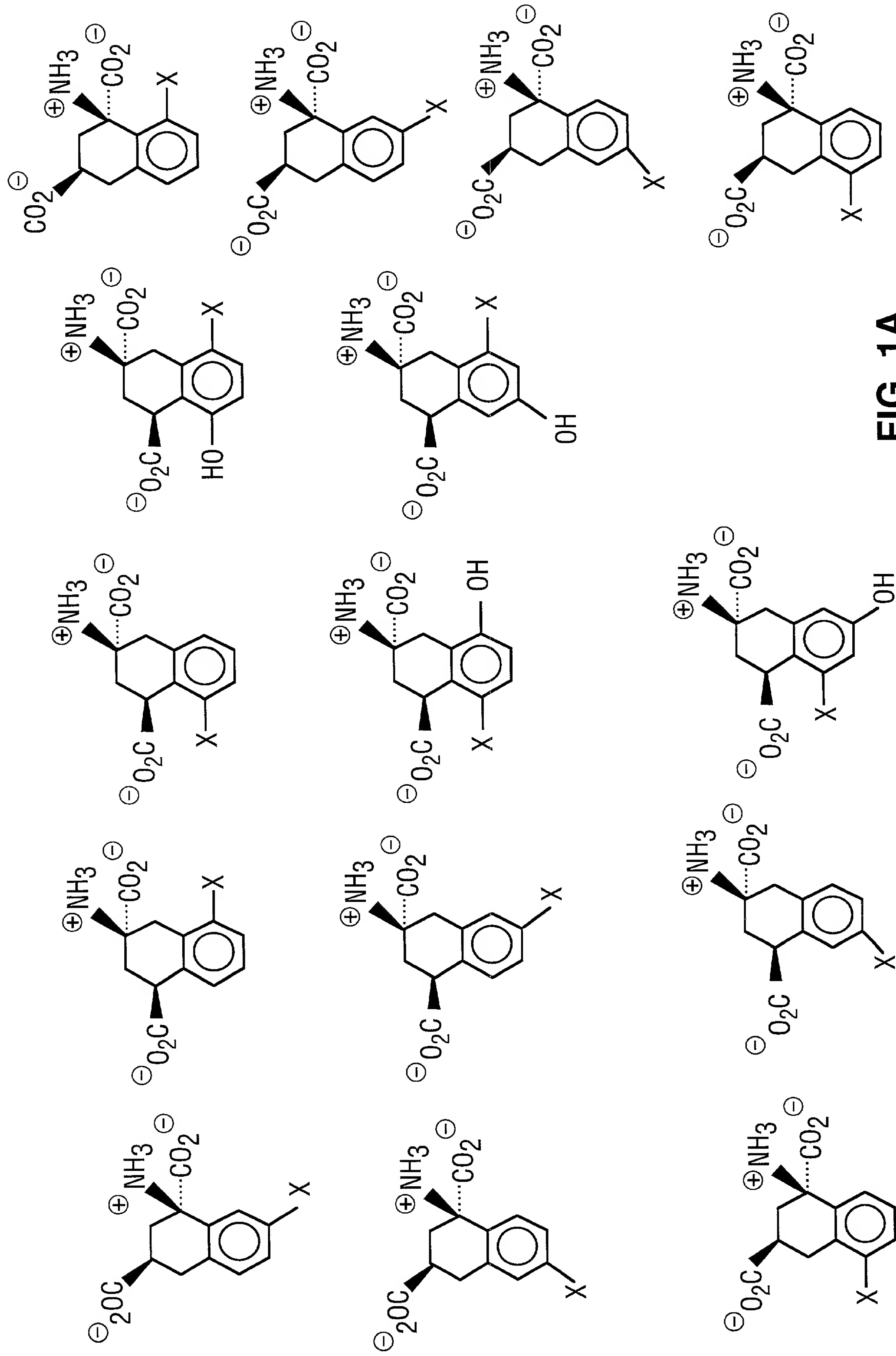


FIG. 1A

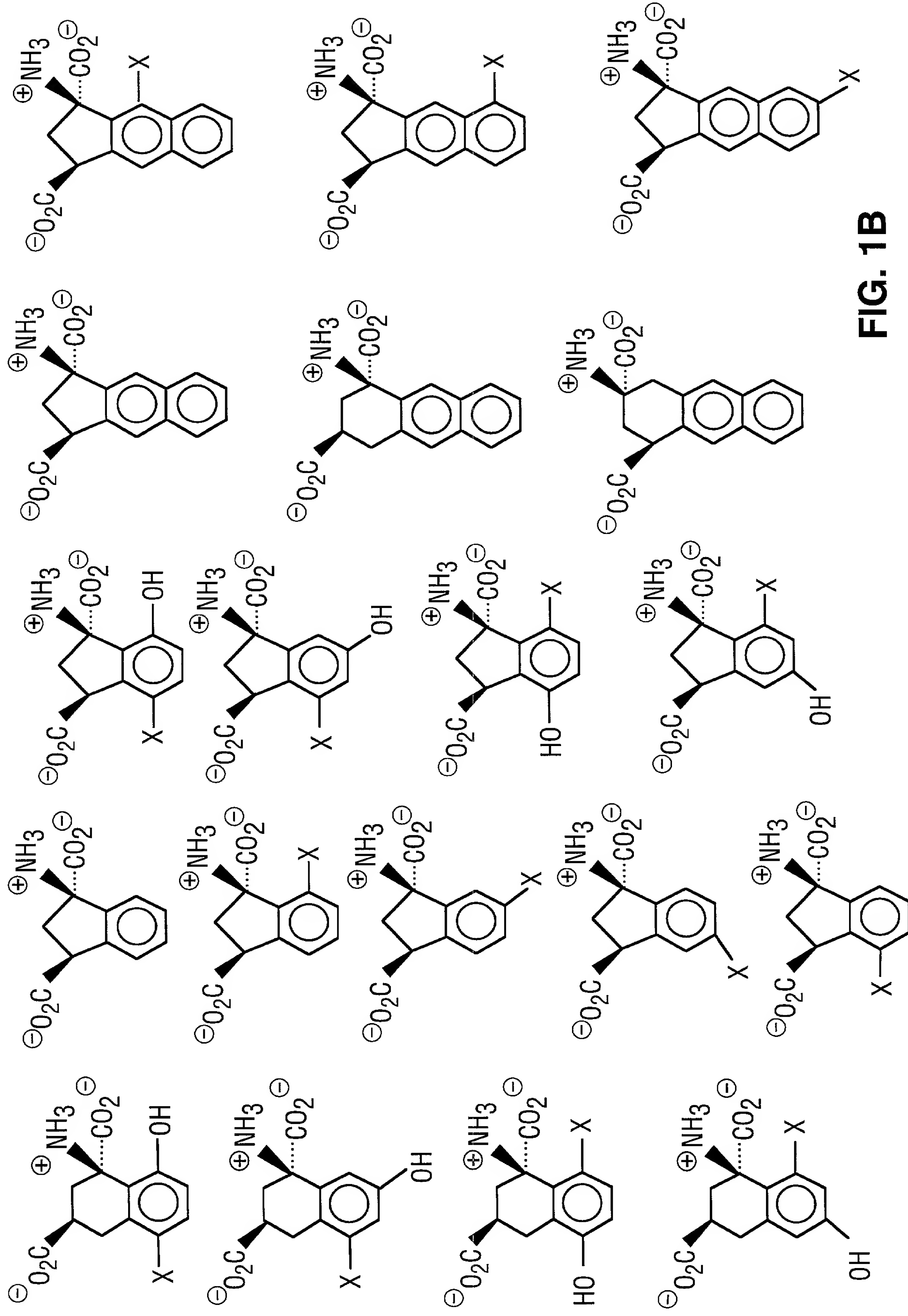


FIG. 1B

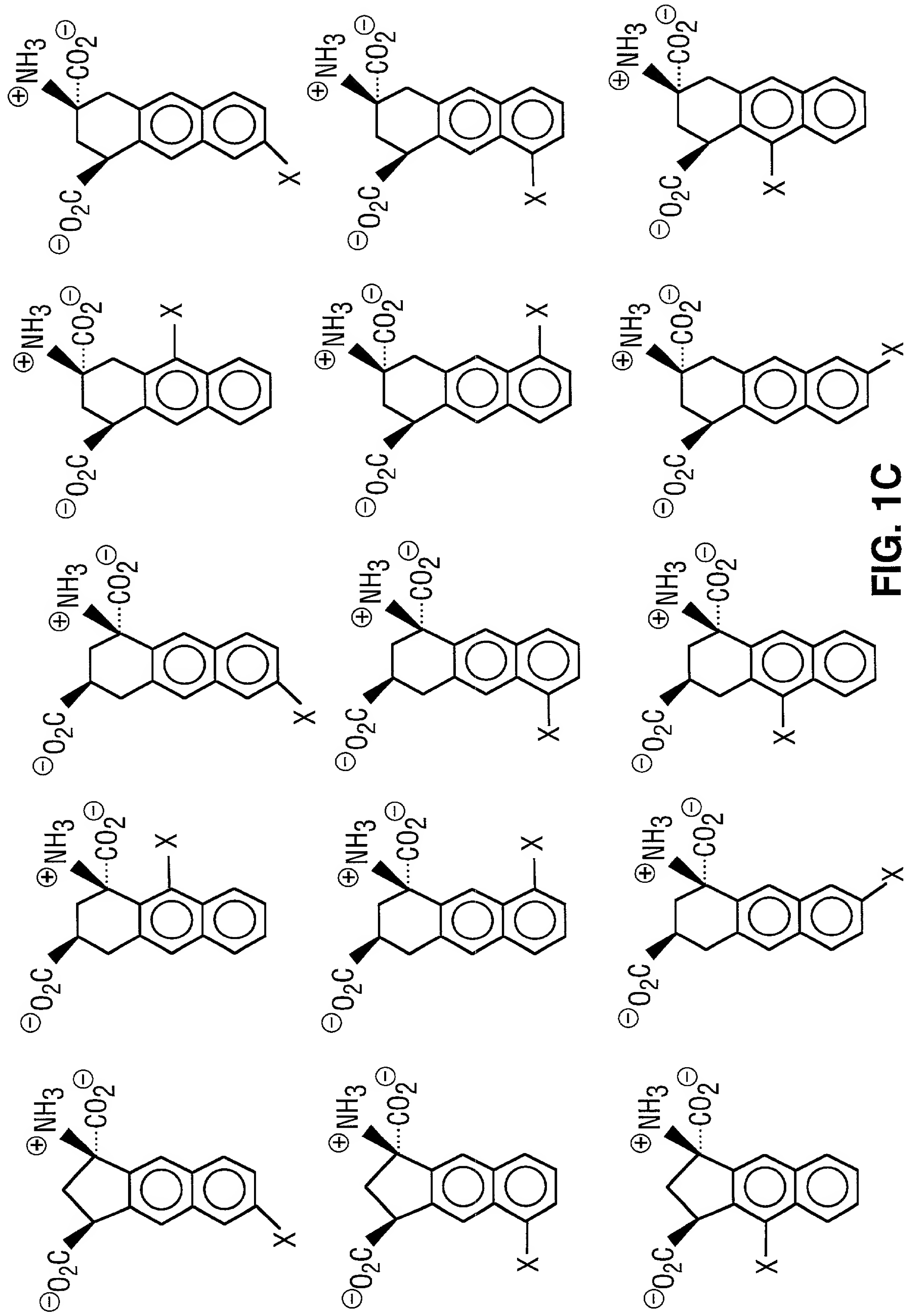


FIG. 1C

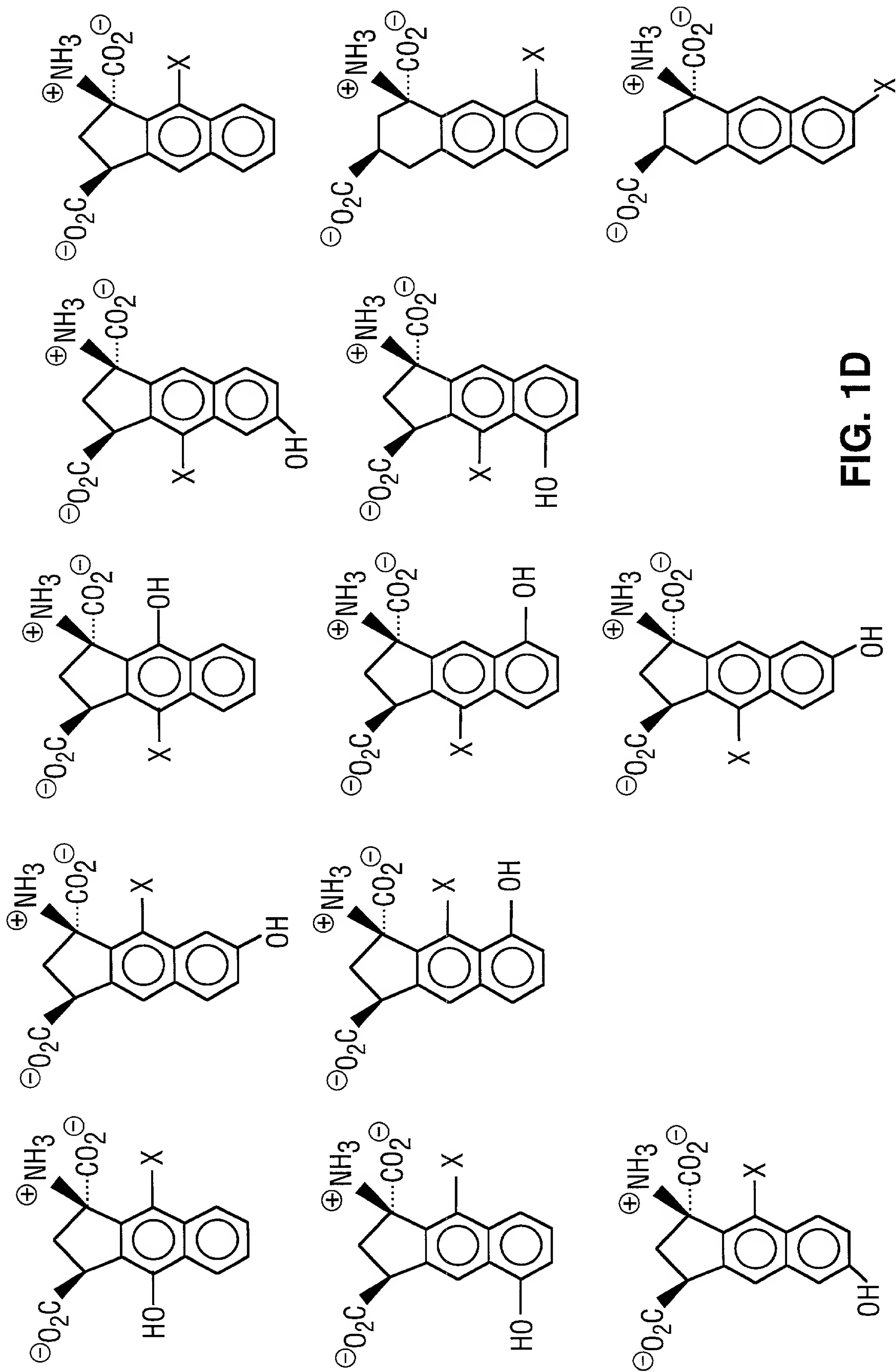


FIG. 1D

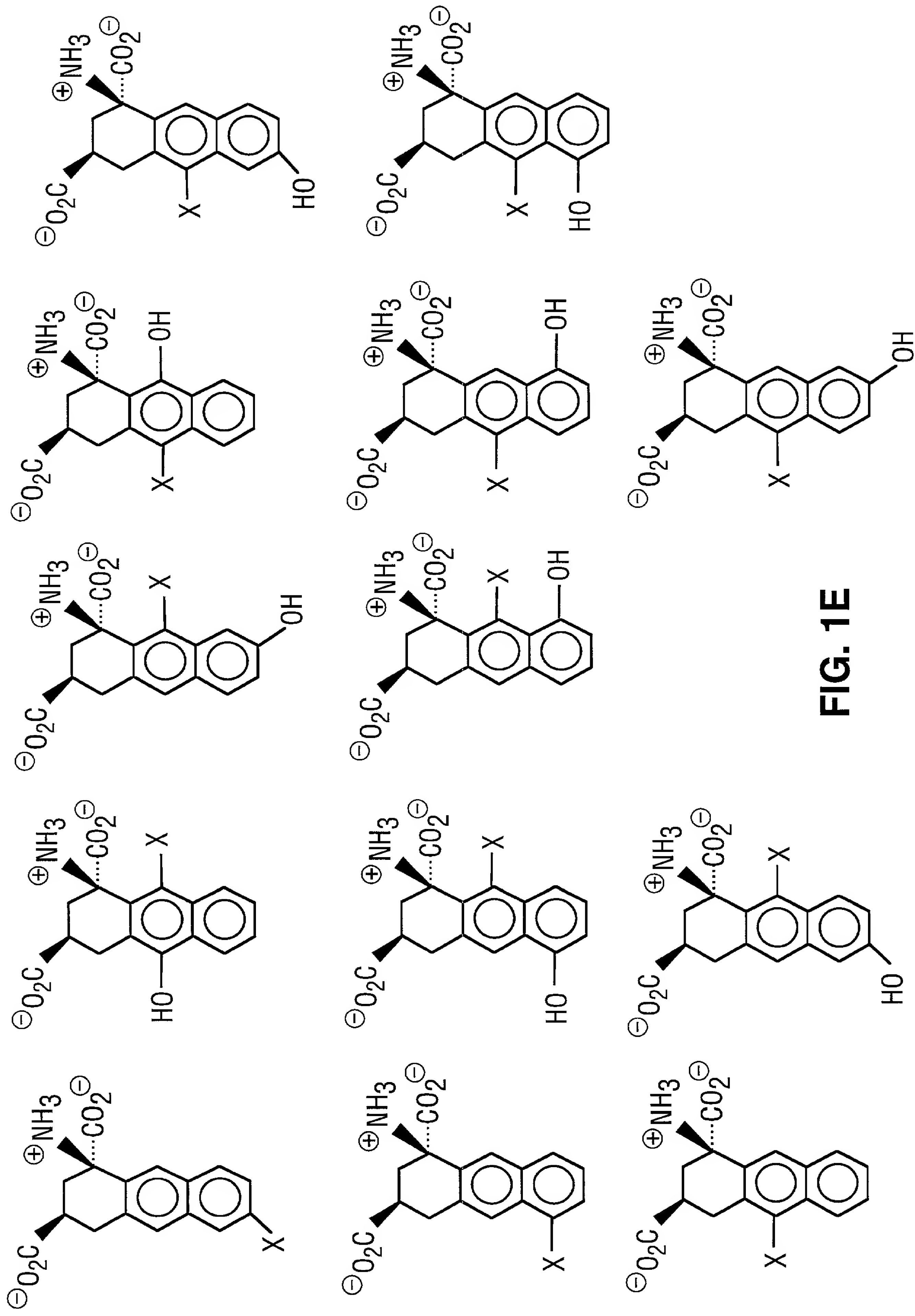


FIG. 1E

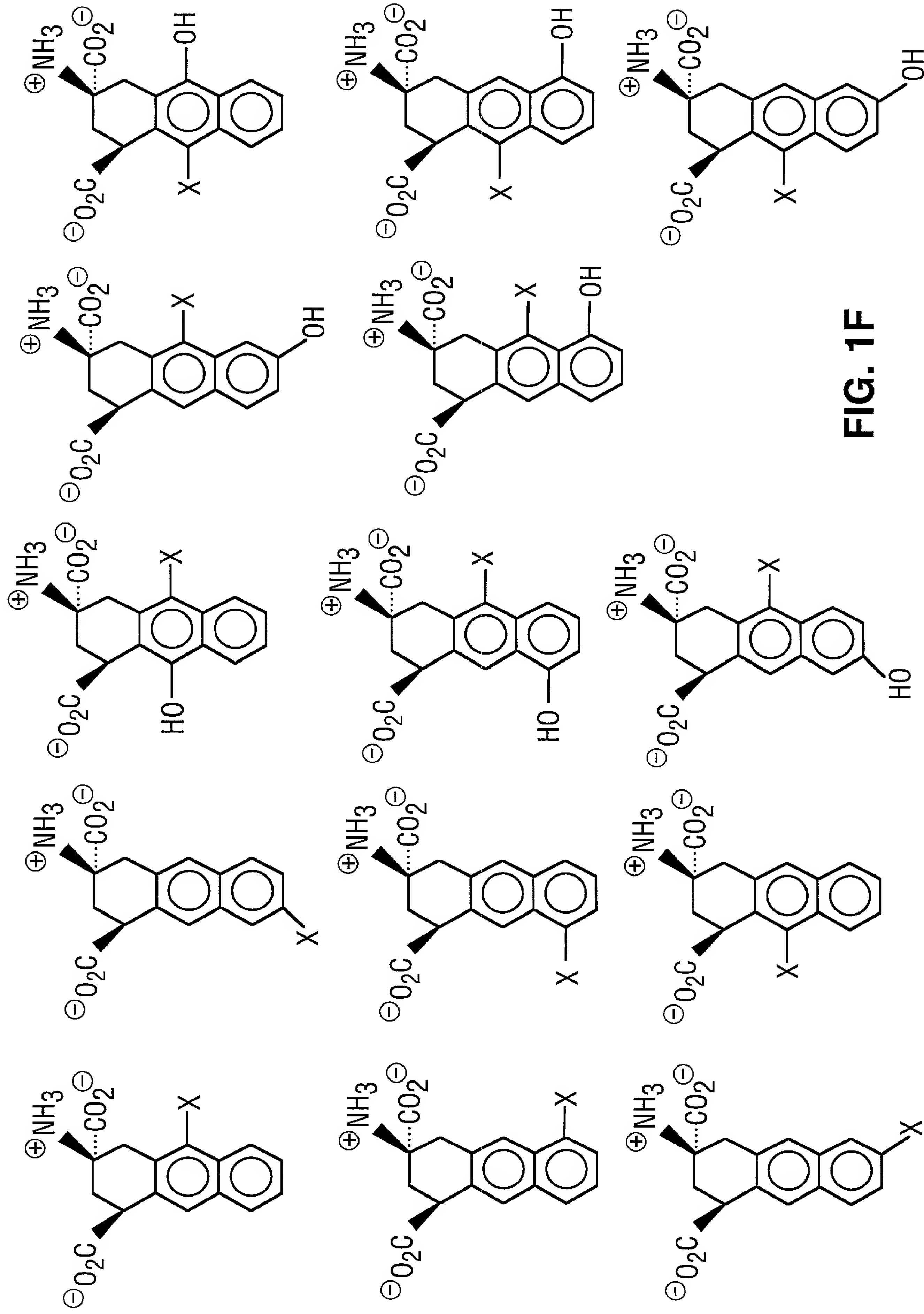


FIG. 1F

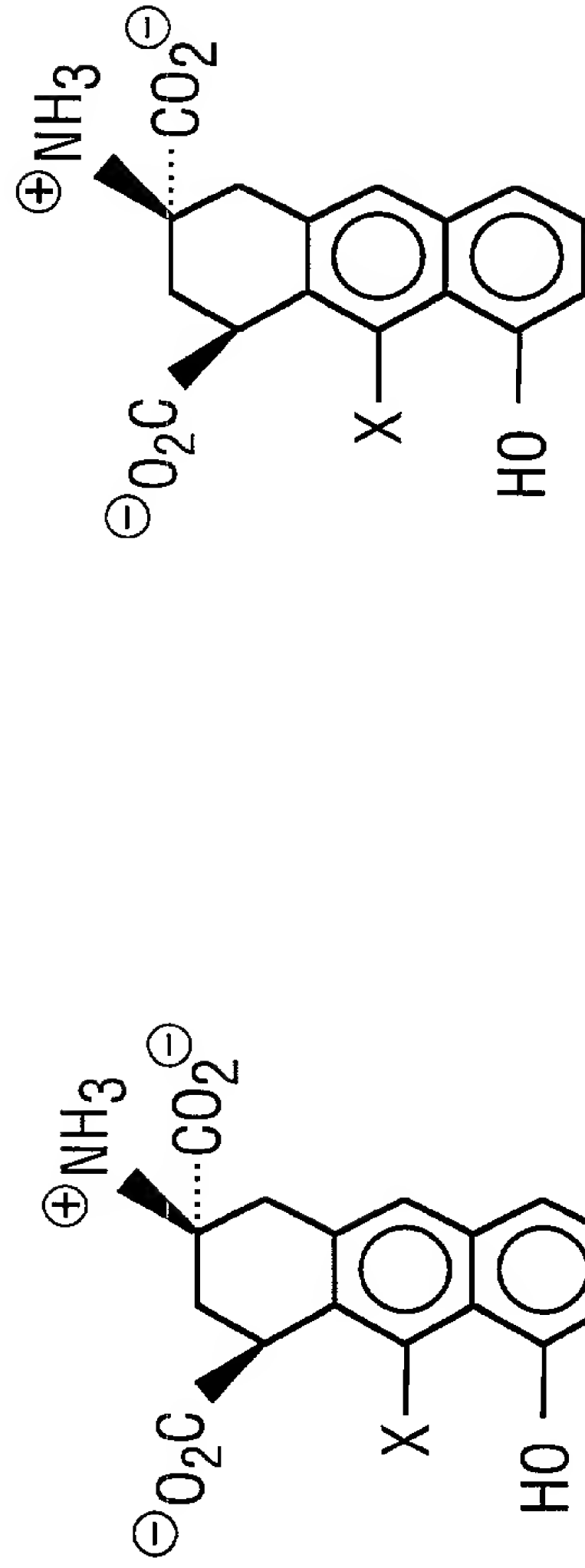
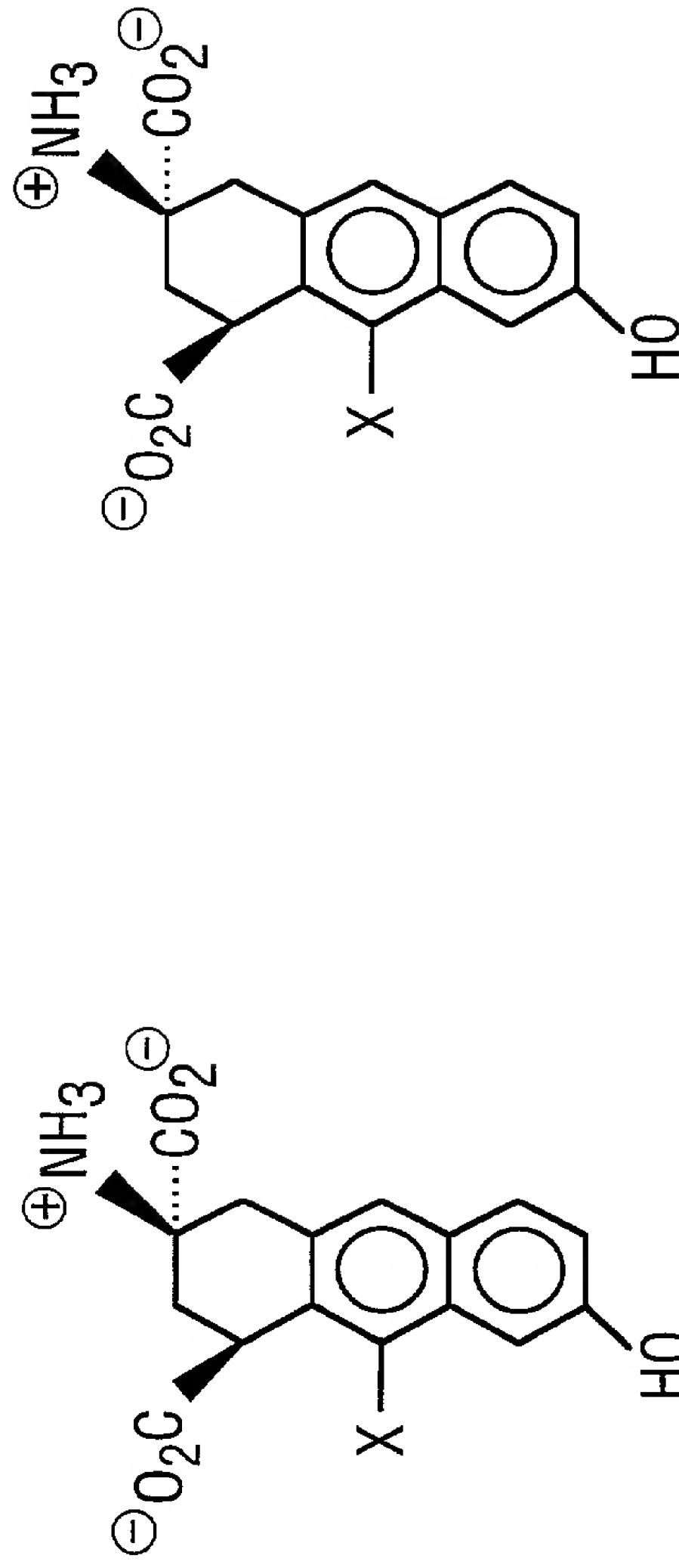


FIG. 1G

FIG. 2

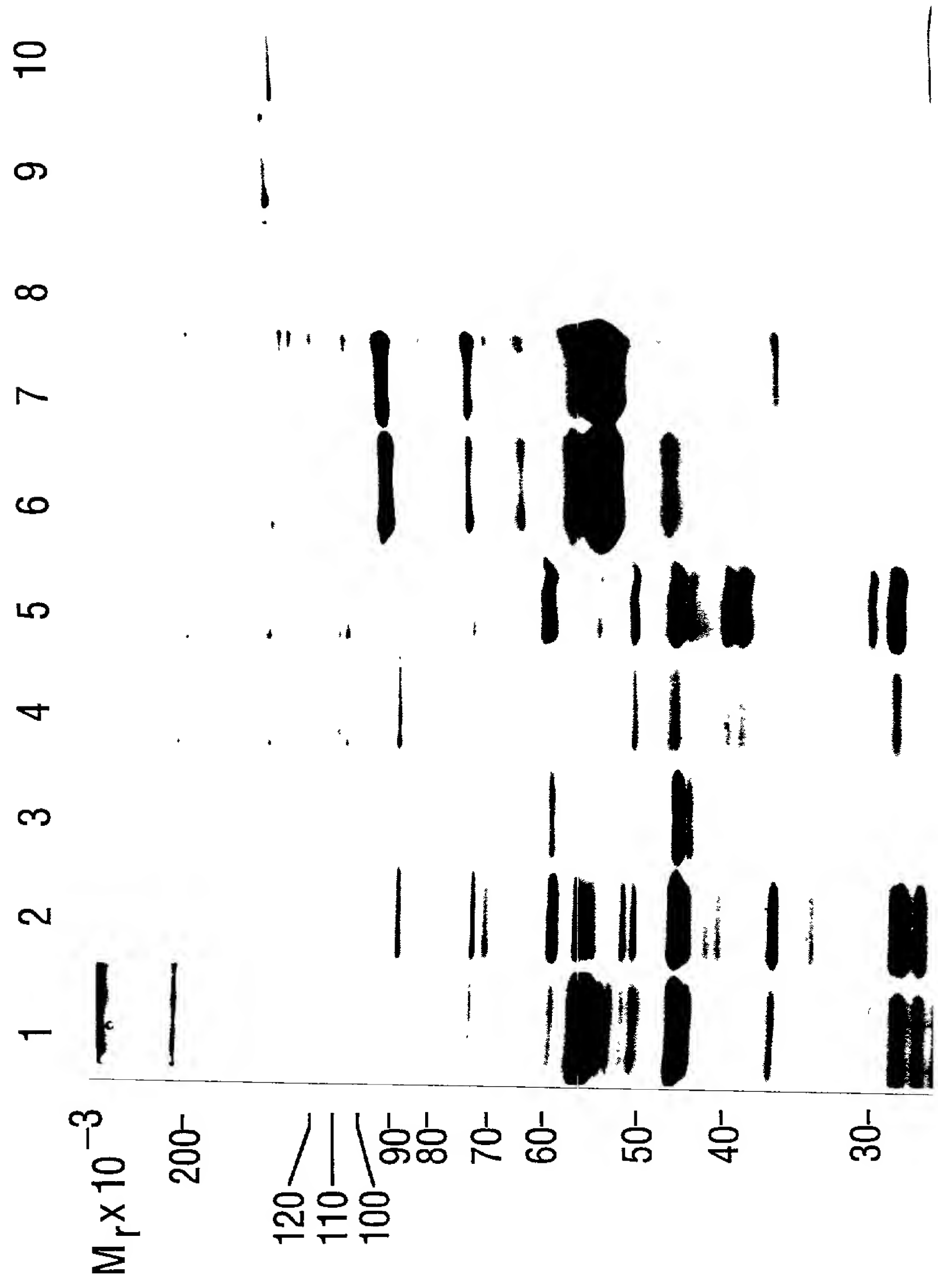


FIG. 2

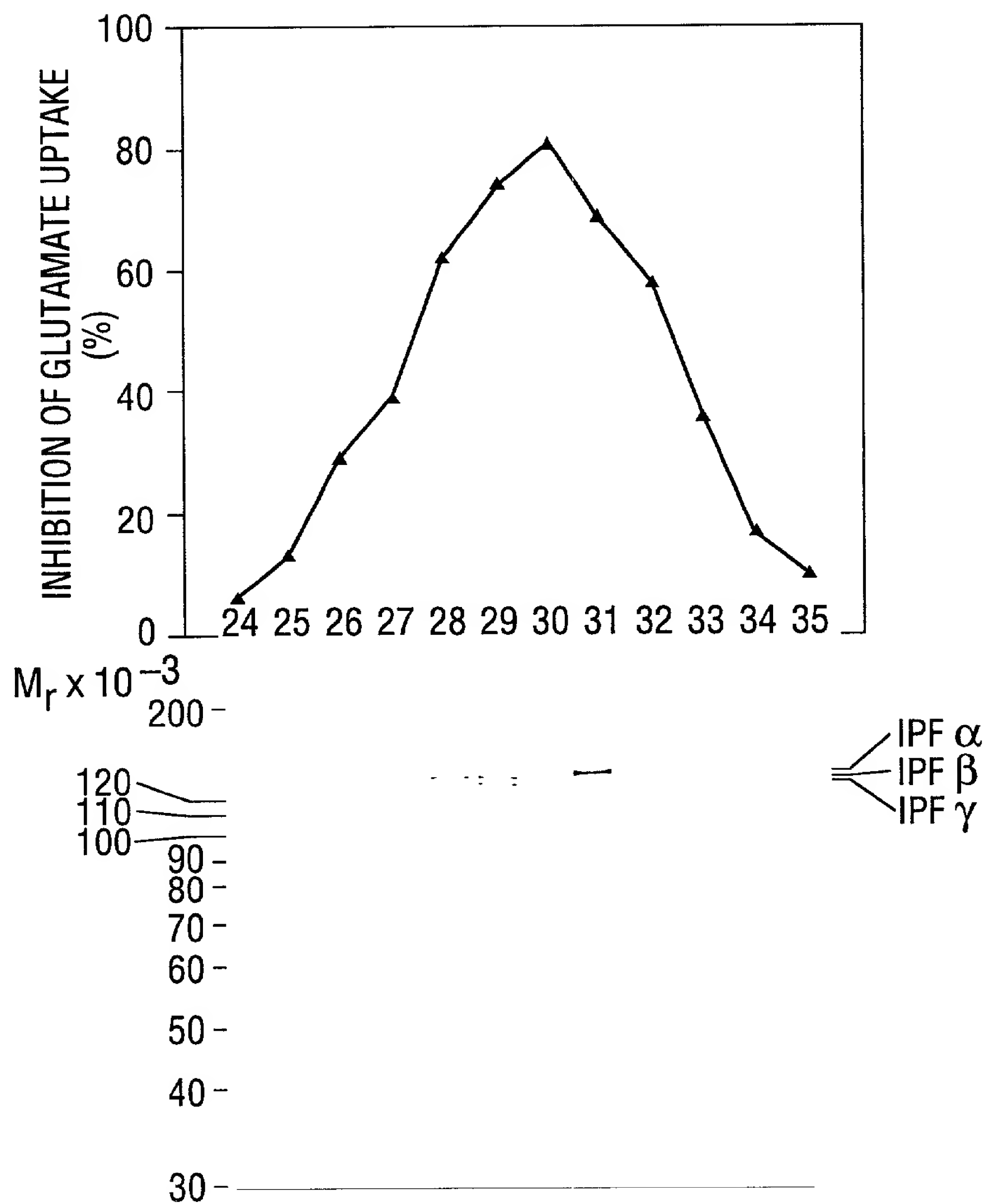


FIG. 3

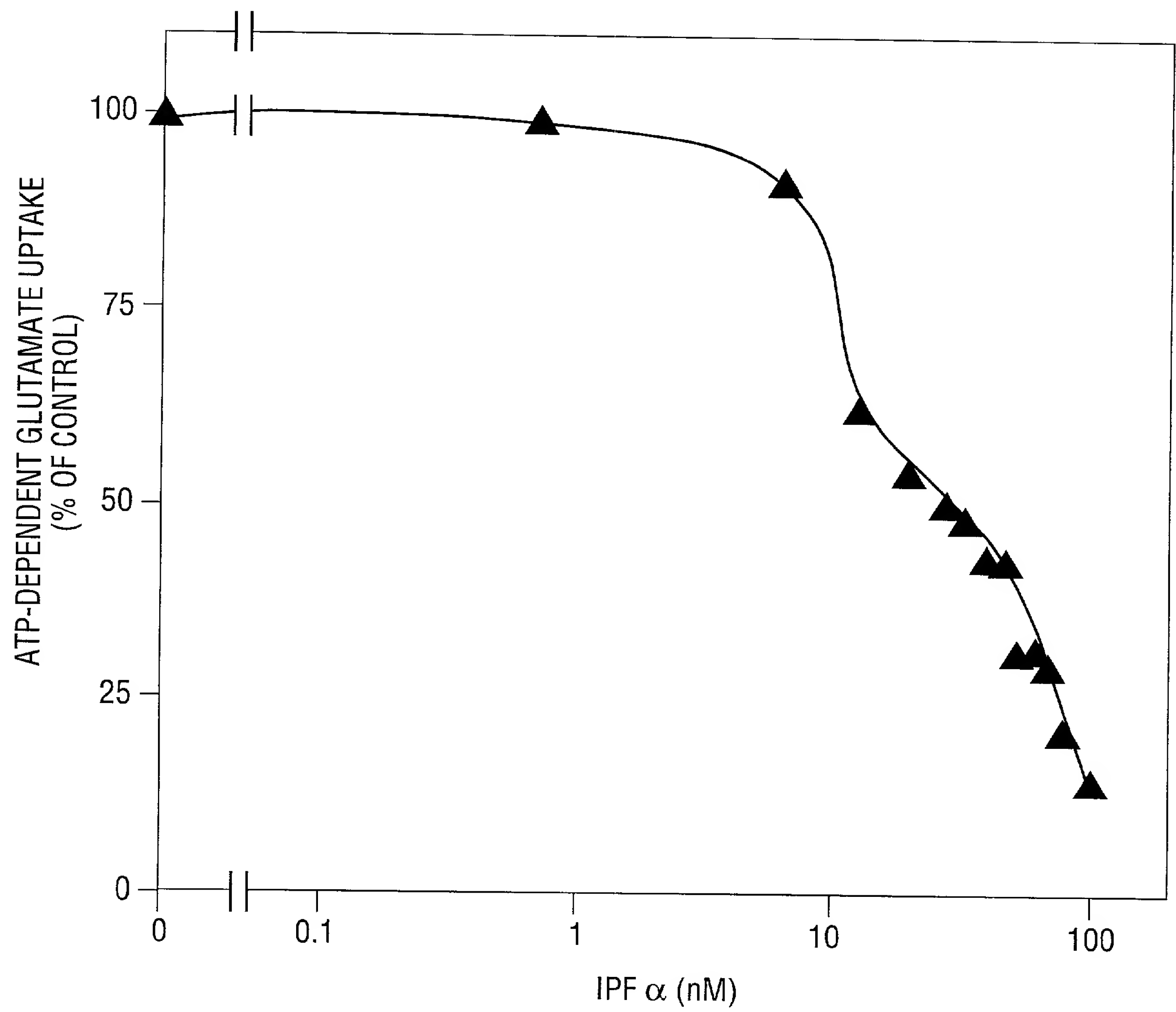


FIG. 4

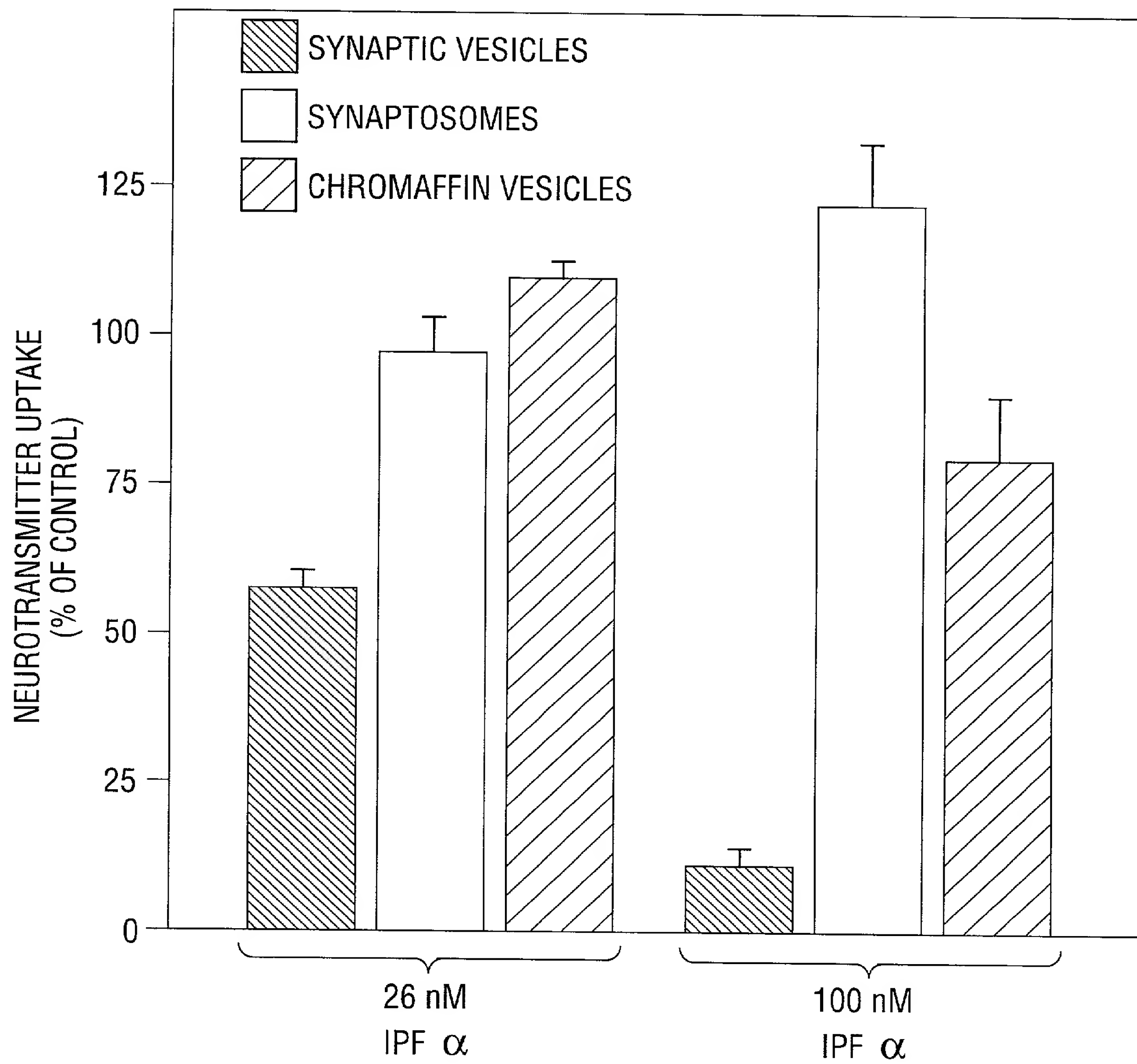


FIG. 5

1 MDPSGVKVL TAEDIQERRQ QVLD**RYHRFK** **ELSTLRRQKL** **EDSYRFQFFQ** RDAEELEKWI
61 QEKLIASDE NYKDPTNLQG KLOYHQAFEA EVQANS GAIV KLDETGNLMI SEGHFASETI
121 RTRLMEHHRQ WELMLEKMR KGIKLLQAQN LVQYLRECE VMDWINDKEA IVTSEELGQD
181 LEHVEVLQKK FEEFQTDMAA HEERVNEVNQ FAAKLIQEQH PEEELIKTKQ DEVNAAWQRL
241 KGLALQRQGG LFGAAEVQRF NRDVDETI SW I KEKEQLMAS DDFGRDLASV QALLRKHEGL
301 ERDLAALEDK VKALCAEADR LQQSHPLSAT QIQVKREELI TNWEQIRTLA AERHARLND S
361 YRLQRFLADF RDLTSWVTEM KALINADELA SDVAGAEALL DRHQEHKGEI DAHEDSFK**SA**
421 **DESDQALLAA** **GHYASDEVRE** KLTVLSEERA ALLELWELRR QQYEQCMDLQ LFYRDTEQVD
481 NWMSKQEAFL LNEDLGDFLD SVEALLKKHE DFEKSLSAQE EKITALDEFA TKLIQNNHYA
541 MEDVATRRDA LLSRRNALHE RAMRRRAQLA DSFHLQQFFR DSDELKSWVN EKMKTATDEA
601 YKDPSNLQGG VQKHQAFEA LSANQSRIDA LEKAGQKLID VNHYAK**DEVA** **ARMNEVISLW**
661 **KKLLEATELK** GIKLREANQQ QQFNRNVEDI ELWLYEVEGH LASDDYGGKDL TNVQNLQKKH
721 ALLEADVAAH QDRIDGVTIQ ARQFDAGHF DAENIKKKQE ALVARYEALK EPMVARKQKI
781 ADSLRQLQLF RDVEDEETWI REKEPIAAST NRGKDLIGVQ NLLKKHQALQ AEIAGHEPRI
841 KAVTQKGNAM VEEGHFAAED VKAKLHELNQ KWEALKAKAS QRRQDLED SL QAQQYFADAN
901 EAESWMREKE PIVGSTDYGG DEDSAEALLK KHEALMSDLS AYGSSIQALR EQAQSCRQQV
961 APTDDETGKE LVLALDYQE KSPREVTMKK **GDILTLLNST** NKDWWKVEVN DRQGFVPAAY
1021 VKKLDPAQSA SRENLL EEQG SIALRQEID NQTRITKEAG SVSLRMKQVE ELYHSLLELG
1081 EKRXGMLEKS CKKFMLFREA NELQQWINEK **EAALTSEEVG** ADLEQVEVLQ KKFDDDFQKDL
1141 KANESRLKDI NKVAEDLESE GLMAEEVQAV QQQEV**YGMMP** RDETD SKTAS PWKSARLMVH

FIG. 6A

1			YHRFK	ELSTLRRQKL	EDSYRFQFFQ	RDAEELEKWI
36	QEKLIQIASDE	NYKDPTNLQG	KLQKHQAFEA	EVQANSQAIV	KLDETGNLMI	SEGHFASETI
96	RTRLMEHLRQ	WELLLEKMRE	KGIKLLQAQN	LVQYLRECE	VMDWINDKEA	IVTSEELGQD
156	LEHVEVLQKK	FEFQTDMAA	HEERVNEVNQ	FAAKLIQEQH	PEEELIKTKQ	DEVNAAWQRL
216	KGLALQRQ GK	LFGAAEVQRF	NRDVDETISW	IKEKEQLMAS	DDFGRDLASV	QALLRKHEGL
276	ERDLAALEDK	VKALCAEADR	LQQSHPLSAT	QIQVKREELI	TNWEQIRTLA	AERHARLND
336	YRLQRFLADF	RDLTSWVTEM	KALINADELA	SDVAGAEALL	DRHQEHKGEI	DAHEDSFKSA
396	DESGQALLAA	GHYASDEVRE	KLTVLSEERA	ALLELWELRR	QQYEQCMDLQ	LFYRDTQEQD
456	NWMSKQEAFL	LNEDLGDFLD	SVEALLKKHE	DFEKSLSAQE	EKITALDEFA	TKLIQNNHYA
516	MEDVATRRDA	LLSRRNALHE	RAMRRRAQLA	DSFHLQQFFR	DDELKSWVN	EKMKTATDEA
576	YKDPSNLQ GK	VQKHQAFEA	LSANQSRIDA	LEKAGQKLID	VNHYAKDEVA	ARMNEVISLW
636	KLLLEATELK	GIKLREANQQ	QQFNRNVEDI	ELWLYEVEGH	LASDDYGKDL	TNVQNLQKKH
696	ALLEADVAAH	QDRIDGVTIQ	ARQFQDAGHF	DAENIKKKQE	ALVARYEALK	EPMVARKQKL
956	ADSLRLQQLF	RDVEDEETWI	REKEPIAAS	NRGKDLIGVQ	NLLKKHQALQ	AEIAGHEPRI
1016	KAVTQKGNAM	VEEGHFAAED	VKAKLHELNQ	KWEALKAKAS	QRRQDLED	SLQAQQYFADAN
1076	EAESWMREKE	PIVGSTDY GK	DEDSAEALLK	KHEALMSDLS	AYGSSIQALR	EQAQSCRQQV
1136	APTDDDETGKE	LVLALYDYQE	KSPREVTM KK	GDILTLLNST	NKDWKVEVN	DRQGFVPAAY
1196	VKKLDPAQSA	SRENLL EEQ	SIALRQEQID	NQTRITKEAG	SVSLRMKQVE	ELYHSLLELG
1256	EKRKGMLEKS	CKKFMLFREA	NELQQWINEK	EAALTSEEVG	ADLEQVEVLQ	KKFDDFQKDL
1316	KANESRLKDI	NKVAEDLESE	GLMAEEVQAV	QQQEVY		

FIG. 6B

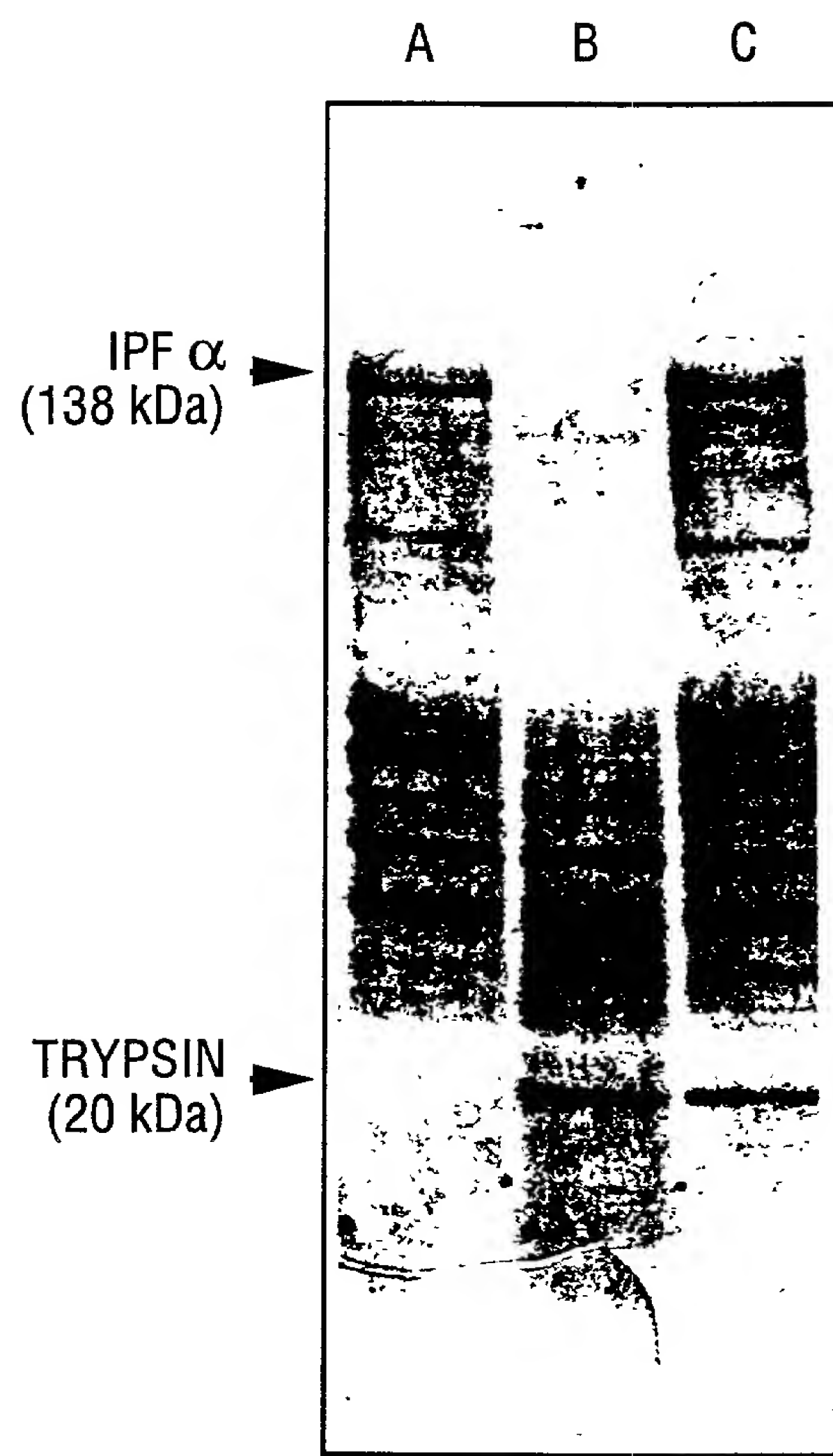


FIG. 7

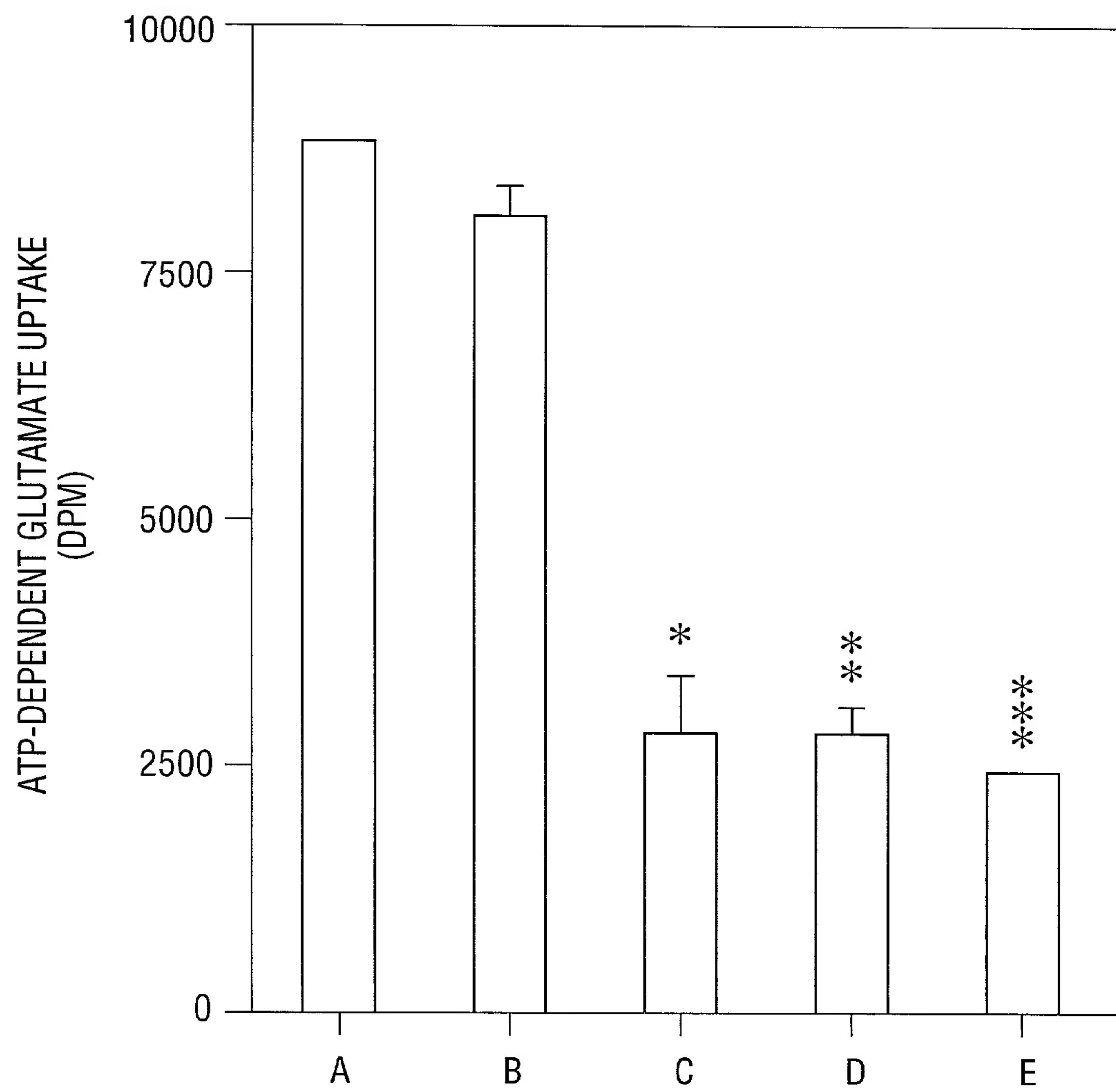


FIG. 8

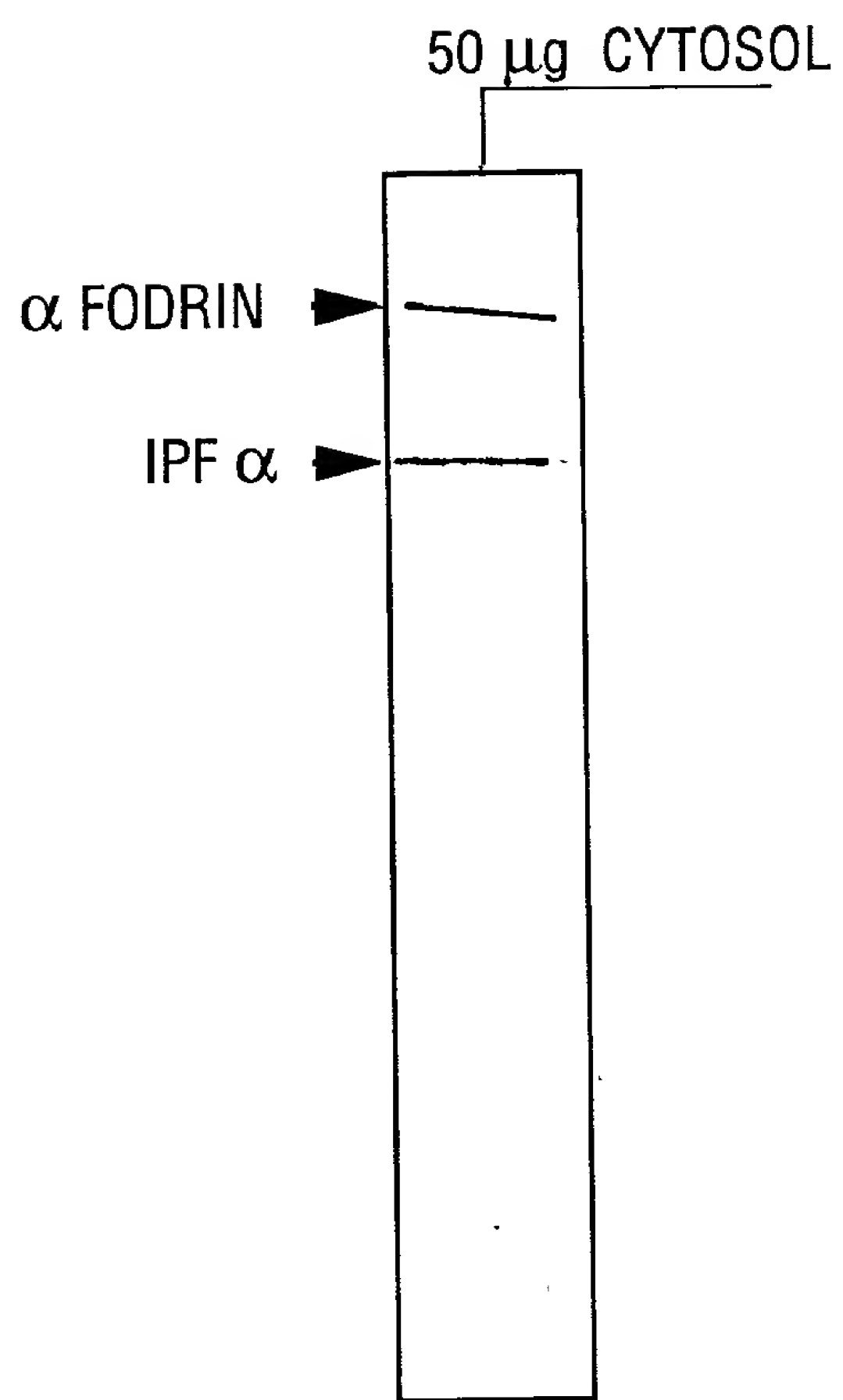


FIG. 9A

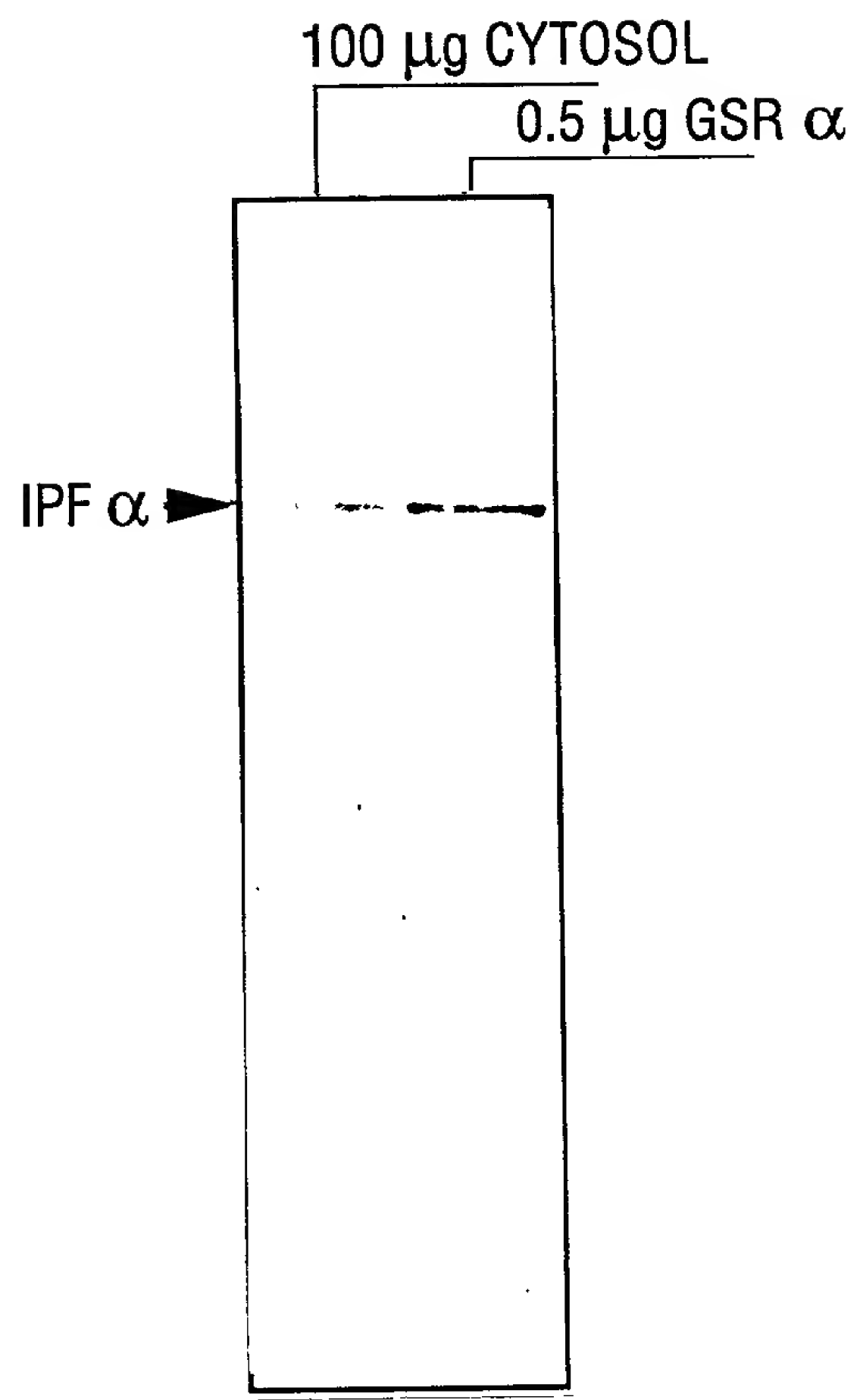


FIG. 9B

DECLARATION FOR PATENT APPLICATION


As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name. I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled **COMPOSITIONS AND METHODS FOR THE INHIBITION OF NEUROTRANSMITTER UPTAKE OF SYNAPTIC VESICLES**, the specification of which was filed on **04/15/97** as Application Serial No. **08/840,006**. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.


I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Sole or First Inventor: **Tetsufumi Ueda**

Inventor's Signature: <u></u>	Date: <u>6-12-97</u>
Residence: <u>3474 Richmond Court, Ann Arbor, Michigan 48105</u>	Citizenship: <u>Japan</u>
Post Office Address: <u>3474 Richmond Court, Ann Arbor, Michigan 48105</u>	

Full Name of Second Joint Inventor: **Eric D. Özkan**

Inventor's Signature: <u></u>	Date: <u>6-12-97</u>
Residence: <u>1505 Roslyn Road, Grosse Pointe Woods, Michigan 48236-1054</u>	Citizenship: <u>United States of America</u>
Post Office Address: <u>1505 Roslyn Road, Grosse Pointe Woods, Michigan 48236-1054</u>	



PATENT
Attorney Docket No.: **UM-02786**
UM File No.: 1342

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Tetsufumi Ueda *et al.* Group No.:
Serial No.: 08/840,006 Examiner:
Filed: 04/15/97
Entitled: **COMPOSITIONS AND METHODS FOR THE INHIBITION OF
NEUROTRANSMITTER UPTAKE OF SYNAPTIC VESICLES**

POWER OF ATTORNEY BY ASSIGNEE

Assistant Commissioner for Patents
Washington, D.C. 20231

THE REGENTS OF THE UNIVERSITY OF MICHIGAN, as Assignee of record of the entire interest of the above-identified patent application, hereby appoints the members of the firm of MEDLEN & CARROLL, LLP, a firm composed of:

Virginia S. Medlen	(Reg. No. 32,050)
Peter G. Carroll	(Reg. No. 32,837)
Kamrin T. MacKnight	(Reg. No. 38,230)
Diane E. Ingolia	(Reg. No. 40,027)
Donald W. Wyatt	(Reg. No. P-40,879)

as its attorneys with full power of substitution to prosecute this application and transact all business in the Patent and Trademark Office in connection therewith.

Please direct all future correspondence and telephone calls regarding this application to:

Donald W. Wyatt	
MEDLEN & CARROLL, LLP	
220 Montgomery Street, Suite 2200	Telephone: 415/705-8410
San Francisco, California 94104	Facsimile: 415/397-8338

I hereby certify that the Assignment document filed with the application or filed subsequent to the filing date of the application, has been reviewed and I hereby certify that, to the best of my knowledge and belief, title is with THE REGENTS OF THE UNIVERSITY OF MICHIGAN.

Dated: 6-16-97

By: 
Name: Robert L. Robb

Title: Director, Technology Management Office
THE REGENTS OF THE UNIVERSITY OF MICHIGAN
3003 South State Street
Ann Arbor, Michigan 48109-1280

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Tetsufumi Ueda *et al.*

Serial No.: not yet assigned

Group No.: not yet assigned

Filed: not yet assigned

Examiner: not yet assigned

Entitled: **Compositions And Methods For The Inhibition
Of Neurotransmitter Uptake Of Synaptic
Vesicles**

CERTIFICATE RE: SEQUENCE LISTING

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.10

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL 079 497 037 US, addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: July 10, 2000

By: 

Anne M. Neiswander

Sir or Madam:

I hereby state that the enclosed Sequence Listing is being submitted in paper copy only (copy on diskette was previously submitted in parent application) and the content of the paper and computer readable copies (as previously submitted in parent application) are the same.

Dated: July 10, 2000

By: 

Kamrin T. MacKnight
Registration No. 38,230

Please direct all communication to:

Peter G. Carroll
Registration No. 32,837
MEDLEN & CARROLL, LLP
220 Montgomery Street, Suite 2200
San Francisco, California 94104
415.705.8410

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Tetsufumi Ueda *et al.*

Serial No.: To be assigned

Group No.: To be assigned

Filed: Herewith

Examiner: To be assigned

Entitled: **COMPOSITIONS AND METHODS FOR THE
INHIBITION OF NEUROTRANSMITTER
UPTAKE OF SYNAPTIC VESICLES**

**REQUEST UNDER 37 C.F.R. § 1.821(e)
TO USE COMPUTER READABLE FORM
FROM ANOTHER APPLICATION**

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated:

07/10/2000

By:


Anne M. Neiswander


Sir or Madam:

The computer readable form in the instant application is identical with that filed in a prior application, Serial Number 08/840,006 filed on April 15, 1997. In accordance with 37 CFR § 1.821(e), please use the computer readable form in the prior application, as the computer readable form for the instant application.

It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application.

The paper copy of the "Sequence Listing" in the instant application is identical to the computer readable copy filed for the prior application.

Dated: 10 July 2000

By: 
Kamrin T. MacKnight
Registration No. 32,837
MEDLEN & CARROLL, LLP
220 Montgomery Street, Suite 2200
San Francisco, California 94104
415.705.8410

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Ueda, Tetsufumi
Ozkan, Eric D.
- (ii) TITLE OF INVENTION: Compositions And Methods For The
Inhibition Of Neurotransmitter Uptake Of Synaptic Vesicles
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Medlen & Carroll, LLP
 - (B) STREET: 220 Montgomery Street, Suite 2200
 - (C) CITY: San Francisco
 - (D) STATE: California
 - (E) COUNTRY: United States of America
 - (F) ZIP: 94104
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/840,006
 - (B) FILING DATE: 15-APR-1997
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Carroll, Peter G.
 - (B) REGISTRATION NUMBER: 32,837
 - (C) REFERENCE/DOCKET NUMBER: UM-02786
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (415) 705-8410
 - (B) TELEFAX: (415) 397-8338

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: not relevant
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Glu	Ala	Ala	Leu	Thr	Ser	Glu	Glu	Val	Gly
1				5					10

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 5 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr His Arg Phe Lys
1 5

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Tyr His Arg Phe Lys Glu Leu Ser Thr Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Tyr His Arg Phe Lys Glu Leu Ser Thr Leu Arg Arg Gln Lys Leu Glu
1 5 10 15

Asp Ser Tyr Arg
 20

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1200 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met	Asp	Pro	Ser	Gly	Val	Lys	Val	Leu	Glu	Thr	Ala	Glu	Asp	Ile	Gln	
1				5					10					15		
Glu	Arg	Arg	Gln	Gln	Val	Leu	Asp	Arg	Tyr	His	Arg	Phe	Lys	Glu	Leu	
			20					25					30			
Ser	Thr	Leu	Arg	Arg	Gln	Lys	Leu	Glu	Asp	Ser	Tyr	Arg	Phe	Gln	Phe	
		35					40					45				
Phe	Gln	Arg	Asp	Ala	Glu	Glu	Leu	Glu	Lys	Trp	Ile	Gln	Glu	Lys	Leu	
	50					55					60					
Gln	Ile	Ala	Ser	Asp	Glu	Asn	Tyr	Lys	Asp	Pro	Thr	Asn	Leu	Gln	Gly	
65					70					75					80	
Lys	Leu	Gln	Lys	His	Gln	Ala	Phe	Glu	Ala	Glu	Val	Gln	Ala	Asn	Ser	
				85					90					95		
Gly	Ala	Ile	Val	Lys	Leu	Asp	Glu	Thr	Gly	Asn	Leu	Met	Ile	Ser	Glu	
			100					105					110			
Gly	His	Phe	Ala	Ser	Glu	Thr	Ile	Arg	Thr	Arg	Leu	Met	Glu	Leu	His	
		115					120					125				
Arg	Gln	Trp	Glu	Leu	Leu	Leu	Glu	Lys	Met	Arg	Glu	Lys	Gly	Ile	Lys	
						135					140					
Leu	Leu	Gln	Ala	Gln	Asn	Leu	Val	Gln	Tyr	Leu	Arg	Glu	Cys	Glu	Asp	
145					150					155					160	
Val	Met	Asp	Trp	Ile	Asn	Asp	Lys	Glu	Ala	Ile	Val	Thr	Ser	Glu	Glu	
				165					170					175		
Leu	Gly	Gln	Asp	Leu	Glu	His	Val	Glu	Val	Leu	Gln	Lys	Lys	Phe	Glu	
			180					185					190			
Glu	Phe	Gln	Thr	Asp	Met	Ala	Ala	His	Glu	Glu	Arg	Val	Asn	Glu	Val	
		195					200					205				
Asn	Gln	Phe	Ala	Ala	Lys	Leu	Ile	Gln	Glu	Gln	His	Pro	Glu	Glu	Glu	
		210				215					220					
Leu	Ile	Lys	Thr	Lys	Gln	Asp	Glu	Val	Asn	Ala	Ala	Trp	Gln	Arg	Leu	
225					230					235					240	
Lys	Gly	Leu	Ala	Leu	Gln	Arg	Gln	Gly	Lys	Leu	Phe	Gly	Ala	Ala	Glu	
				245					250					255		
Val	Gln	Arg	Phe	Asn	Arg	Asp	Val	Asp	Glu	Thr	Ile	Ser	Trp	Ile	Lys	
			260					265					270			
Glu	Lys	Glu	Gln	Leu	Met	Ala	Ser	Asp	Asp	Phe	Gly	Arg	Asp	Leu	Ala	
		275					280					285				
Ser	Val	Gln	Ala	Leu	Leu	Arg	Lys	His	Glu	Gly	Leu	Glu	Arg	Asp	Leu	
		290				295					300					
Ala	Ala	Leu	Glu	Asp	Lys	Val	Lys	Ala	Leu	Cys	Ala	Glu	Ala	Asp	Arg	
305					310					315					320	

Leu	Gln	Gln	Ser	His	Pro	Leu	Ser	Ala	Thr	Gln	Ile	Gln	Val	Lys	Arg	
				325					330					335		
Glu	Glu	Leu	Ile	Thr	Asn	Trp	Glu	Gln	Ile	Arg	Thr	Leu	Ala	Ala	Glu	
			340					345					350			
Arg	His	Ala	Arg	Leu	Asn	Asp	Ser	Tyr	Arg	Leu	Gln	Arg	Phe	Leu	Ala	
		355					360					365				
Asp	Phe	Arg	Asp	Leu	Thr	Ser	Trp	Val	Thr	Glu	Met	Lys	Ala	Leu	Ile	
	370					375					380					
Asn	Ala	Asp	Glu	Leu	Ala	Ser	Asp	Val	Ala	Gly	Ala	Glu	Ala	Leu	Leu	
385					390					395					400	
Asp	Arg	His	Gln	Glu	His	Lys	Gly	Glu	Ile	Asp	Ala	His	Glu	Asp	Ser	
			405						410					415		
Phe	Lys	Ser	Ala	Asp	Glu	Ser	Gly	Gln	Ala	Leu	Leu	Ala	Ala	Gly	His	
			420					425					430			
Tyr	Ala	Ser	Asp	Glu	Val	Arg	Glu	Lys	Leu	Thr	Val	Leu	Ser	Glu	Glu	
		435					440					445				
Arg	Ala	Ala	Leu	Leu	Glu	Leu	Trp	Glu	Leu	Arg	Arg	Gln	Gln	Tyr	Glu	
	450					455					460					
Gln	Cys	Met	Asp	Leu	Gln	Leu	Phe	Tyr	Arg	Asp	Thr	Glu	Gln	Val	Asp	
465					470					475					480	
Asn	Trp	Met	Ser	Lys	Gln	Glu	Ala	Phe	Leu	Leu	Asn	Glu	Asp	Leu	Gly	
				485					490					495		
Asp	Phe	Leu	Asp	Ser	Val	Glu	Ala	Leu	Leu	Lys	Lys	His	Glu	Asp	Phe	
			500					505					510			
Glu	Lys	Ser	Leu	Ser	Ala	Gln	Glu	Glu	Lys	Ile	Thr	Ala	Leu	Asp	Glu	
		515					520					525				
Phe	Ala	Thr	Lys	Leu	Ile	Gln	Asn	Asn	His	Tyr	Ala	Met	Glu	Asp	Val	
	530					535					540					
Ala	Thr	Arg	Arg	Asp	Ala	Leu	Leu	Ser	Arg	Arg	Asn	Ala	Leu	His	Glu	
545					550					555					560	
Arg	Ala	Met	Arg	Arg	Arg	Ala	Gln	Leu	Ala	Asp	Ser	Phe	His	Leu	Gln	
				565					570					575		
Gln	Phe	Phe	Arg	Asp	Ser	Asp	Glu	Leu	Lys	Ser	Trp	Val	Asn	Glu	Lys	
			580					585					590			
Met	Lys	Thr	Ala	Thr	Asp	Glu	Ala	Tyr	Lys	Asp	Pro	Ser	Asn	Leu	Gln	
		595					600					605				
Gly	Lys	Val	Gln	Lys	His	Gln	Ala	Phe	Glu	Ala	Glu	Leu	Ser	Ala	Asn	
	610					615					620					
Gln	Ser	Arg	Ile	Asp	Ala	Leu	Glu	Lys	Ala	Gly	Gln	Lys	Leu	Ile	Asp	
625					630					635					640	
Val	Asn	His	Tyr	Ala	Lys	Asp	Glu	Val	Ala	Ala	Arg	Met	Asn	Glu	Val	
				645					650					655		

Ile	Ser	Leu	Trp	Lys	Lys	Leu	Leu	Glu	Ala	Thr	Glu	Leu	Lys	Gly	Ile	
			660					665					670			
Lys	Leu	Arg	Glu	Ala	Asn	Gln	Gln	Gln	Gln	Phe	Asn	Arg	Asn	Val	Glu	
		675					680					685				
Asp	Ile	Glu	Leu	Trp	Leu	Tyr	Glu	Val	Glu	Gly	His	Leu	Ala	Ser	Asp	
	690					695					700					
Asp	Tyr	Gly	Lys	Asp	Leu	Thr	Asn	Val	Gln	Asn	Leu	Gln	Lys	Lys	His	
	705				710					715					720	
Ala	Leu	Leu	Glu	Ala	Asp	Val	Ala	Ala	His	Gln	Asp	Arg	Ile	Asp	Gly	
				725					730					735		
Val	Thr	Ile	Gln	Ala	Arg	Gln	Phe	Gln	Asp	Ala	Gly	His	Phe	Asp	Ala	
			740					745					750			
Glu	Asn	Ile	Lys	Lys	Lys	Gln	Glu	Ala	Leu	Val	Ala	Arg	Tyr	Glu	Ala	
		755					760					765				
Leu	Lys	Glu	Pro	Met	Val	Ala	Arg	Lys	Gln	Lys	Leu	Ala	Asp	Ser	Leu	
	770					775					780					
Arg	Leu	Gln	Gln	Leu	Phe	Arg	Asp	Val	Glu	Asp	Glu	Glu	Thr	Trp	Ile	
	785				790					795					800	
Arg	Glu	Lys	Glu	Pro	Ile	Ala	Ala	Ser	Thr	Asn	Arg	Gly	Lys	Asp	Leu	
				805					810					815		
Ile	Gly	Val	Gln	Asn	Leu	Leu	Lys	Lys	His	Gln	Ala	Leu	Gln	Ala	Glu	
			820					825					830			
Ile	Ala	Gly	His	Glu	Pro	Arg	Ile	Lys	Ala	Val	Thr	Gln	Lys	Gly	Asn	
		835					840					845				
Ala	Met	Val	Glu	Glu	Gly	His	Phe	Ala	Ala	Glu	Asp	Val	Lys	Ala	Lys	
	850					855					860					
Leu	His	Glu	Leu	Asn	Gln	Lys	Trp	Glu	Ala	Leu	Lys	Ala	Lys	Ala	Ser	
	865				870					875					880	
Gln	Arg	Arg	Gln	Asp	Leu	Glu	Asp	Ser	Leu	Gln	Ala	Gln	Gln	Tyr	Phe	
				885					890					895		
Ala	Asp	Ala	Asn	Glu	Ala	Glu	Ser	Trp	Met	Arg	Glu	Lys	Glu	Pro	Ile	
			900					905					910			
Val	Gly	Ser	Thr	Asp	Tyr	Gly	Lys	Asp	Glu	Asp	Ser	Ala	Glu	Ala	Leu	
		915					920					925				
Leu	Lys	Lys	His	Glu	Ala	Leu	Met	Ser	Asp	Leu	Ser	Ala	Tyr	Gly	Ser	
	930					935					940					
Ser	Ile	Gln	Ala	Leu	Arg	Glu	Gln	Ala	Gln	Ser	Cys	Arg	Gln	Gln	Val	
	945				950					955					960	
Ala	Pro	Thr	Asp	Asp	Glu	Thr	Gly	Lys	Glu	Leu	Val	Leu	Ala	Leu	Tyr	
				965					970					975		
Asp	Tyr	Gln	Glu	Lys	Ser	Pro	Arg	Glu	Val	Thr	Met	Lys	Lys	Gly	Asp	
			980					985					990			

Ile	Leu	Thr	Leu	Leu	Asn	Ser	Thr	Asn	Lys	Asp	Trp	Trp	Lys	Val	Glu	995	1000	1005	
Val	Asn	Asp	Arg	Gln	Gly	Phe	Val	Pro	Ala	Ala	Tyr	Val	Lys	Lys	Leu	1010	1015	1020	
Asp	Pro	Ala	Gln	Ser	Ala	Ser	Arg	Glu	Asn	Leu	Leu	Glu	Glu	Gln	Gly	1025	1030	1035	1040
Ser	Ile	Ala	Leu	Arg	Gln	Glu	Gln	Ile	Asp	Asn	Gln	Thr	Arg	Ile	Thr	1045	1050	1055	
Lys	Glu	Ala	Gly	Ser	Val	Ser	Leu	Arg	Met	Lys	Gln	Val	Glu	Glu	Leu	1060	1065	1070	
Tyr	His	Ser	Leu	Leu	Glu	Leu	Gly	Glu	Lys	Arg	Lys	Gly	Met	Leu	Glu	1075	1080	1085	
Lys	Ser	Cys	Lys	Lys	Phe	Met	Leu	Phe	Arg	Glu	Ala	Asn	Glu	Leu	Gln	1090	1095	1100	
Gln	Trp	Ile	Asn	Glu	Lys	Glu	Ala	Ala	Leu	Thr	Ser	Glu	Glu	Val	Gly	1105	1110	1115	1120
Ala	Asp	Leu	Glu	Gln	Val	Glu	Val	Leu	Gln	Lys	Lys	Phe	Asp	Asp	Phe	1125	1130	1135	
Gln	Lys	Asp	Leu	Lys	Ala	Asn	Glu	Ser	Arg	Leu	Lys	Asp	Ile	Asn	Lys	1140	1145	1150	
Val	Ala	Glu	Asp	Leu	Glu	Ser	Glu	Gly	Leu	Met	Ala	Glu	Glu	Val	Gln	1155	1160	1165	
Ala	Val	Gln	Gln	Gln	Glu	Val	Tyr	Gly	Met	Met	Pro	Arg	Asp	Glu	Thr	1170	1175	1180	
Asp	Ser	Lys	Thr	Ala	Ser	Pro	Trp	Lys	Ser	Ala	Arg	Leu	Met	Val	His	1185	1190	1195	1200

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1151 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Tyr	His	Arg	Phe	Lys	Glu	Leu	Ser	Thr	Leu	Arg	Arg	Gln	Lys	Leu	Glu	1	5	10	15
Asp	Ser	Tyr	Arg	Phe	Gln	Phe	Phe	Gln	Arg	Asp	Ala	Glu	Glu	Leu	Glu	20	25	30	
Lys	Trp	Ile	Gln	Glu	Lys	Leu	Gln	Ile	Ala	Ser	Asp	Glu	Asn	Tyr	Lys	35	40	45	
Asp	Pro	Thr	Asn	Leu	Gln	Gly	Lys	Leu	Gln	Lys	His	Gln	Ala	Phe	Glu	50	55	60	

Ala	Glu	Val	Gln	Ala	Asn	Ser	Gly	Ala	Ile	Val	Lys	Leu	Asp	Glu	Thr	
65					70					75					80	
Gly	Asn	Leu	Met	Ile	Ser	Glu	Gly	His	Phe	Ala	Ser	Glu	Thr	Ile	Arg	
				85					90					95		
Thr	Arg	Leu	Met	Glu	Leu	His	Arg	Gln	Trp	Glu	Leu	Leu	Leu	Glu	Lys	
			100					105					110			
Met	Arg	Glu	Lys	Gly	Ile	Lys	Leu	Leu	Gln	Ala	Gln	Asn	Leu	Val	Gln	
		115					120					125				
Tyr	Leu	Arg	Glu	Cys	Glu	Asp	Val	Met	Asp	Trp	Ile	Asn	Asp	Lys	Glu	
	130					135					140					
Ala	Ile	Val	Thr	Ser	Glu	Glu	Leu	Gly	Gln	Asp	Leu	Glu	His	Val	Glu	
145					150					155					160	
Val	Leu	Gln	Lys	Lys	Phe	Glu	Glu	Phe	Gln	Thr	Asp	Met	Ala	Ala	His	
				165					170					175		
Glu	Glu	Arg	Val	Asn	Glu	Val	Asn	Gln	Phe	Ala	Ala	Lys	Leu	Ile	Gln	
			180					185					190			
Glu	Gln	His	Pro	Glu	Glu	Glu	Leu	Ile	Lys	Thr	Lys	Gln	Asp	Glu	Val	
		195					200					205				
Asn	Ala	Ala	Trp	Gln	Arg	Leu	Lys	Gly	Leu	Ala	Leu	Gln	Arg	Gln	Gly	
	210					215					220					
Lys	Leu	Phe	Gly	Ala	Ala	Glu	Val	Gln	Arg	Phe	Asn	Arg	Asp	Val	Asp	
225				230						235				240		
Glu	Thr	Ile	Ser	Trp	Ile	Lys	Glu	Lys	Glu	Gln	Leu	Met	Ala	Ser	Asp	
				245					250					255		
Asp	Phe	Gly	Arg	Asp	Leu	Ala	Ser	Val	Gln	Ala	Leu	Leu	Arg	Lys	His	
			260					265					270			
Glu	Gly	Leu	Glu	Arg	Asp	Leu	Ala	Ala	Leu	Glu	Asp	Lys	Val	Lys	Ala	
		275					280					285				
Leu	Cys	Ala	Glu	Ala	Asp	Arg	Leu	Gln	Gln	Ser	His	Pro	Leu	Ser	Ala	
	290					295					300					
Thr	Gln	Ile	Gln	Val	Lys	Arg	Glu	Glu	Leu	Ile	Thr	Asn	Trp	Glu	Gln	
305					310					315					320	
Ile	Arg	Thr	Leu	Ala	Ala	Glu	Arg	His	Ala	Arg	Leu	Asn	Asp	Ser	Tyr	
				325					330					335		
Arg	Leu	Gln	Arg	Phe	Leu	Ala	Asp	Phe	Arg	Asp	Leu	Thr	Ser	Trp	Val	
			340					345					350			
Thr	Glu	Met	Lys	Ala	Leu	Ile	Asn	Ala	Asp	Glu	Leu	Ala	Ser	Asp	Val	
		355					360					365				
Ala	Gly	Ala	Glu	Ala	Leu	Leu	Asp	Arg	His	Gln	Glu	His	Lys	Gly	Glu	
	370					375					380					
Ile	Asp	Ala	His	Glu	Asp	Ser	Phe	Lys	Ser	Ala	Asp	Glu	Ser	Gly	Gln	
385					390					395					400	

Ala	Leu	Leu	Ala	Ala	Gly	His	Tyr	Ala	Ser	Asp	Glu	Val	Arg	Glu	Lys
				405					410					415	
Leu	Thr	Val	Leu	Ser	Glu	Glu	Arg	Ala	Ala	Leu	Leu	Glu	Leu	Trp	Glu
			420					425					430		
Leu	Arg	Arg	Gln	Gln	Tyr	Glu	Gln	Cys	Met	Asp	Leu	Gln	Leu	Phe	Tyr
		435					440					445			
Arg	Asp	Thr	Glu	Gln	Val	Asp	Asn	Trp	Met	Ser	Lys	Gln	Glu	Ala	Phe
	450					455					460				
Leu	Leu	Asn	Glu	Asp	Leu	Gly	Asp	Phe	Leu	Asp	Ser	Val	Glu	Ala	Leu
465					470					475					480
Leu	Lys	Lys	His	Glu	Asp	Phe	Glu	Lys	Ser	Leu	Ser	Ala	Gln	Glu	Glu
				485					490					495	
Lys	Ile	Thr	Ala	Leu	Asp	Glu	Phe	Ala	Thr	Lys	Leu	Ile	Gln	Asn	Asn
			500					505					510		
His	Tyr	Ala	Met	Glu	Asp	Val	Ala	Thr	Arg	Arg	Asp	Ala	Leu	Leu	Ser
		515					520					525			
Arg	Arg	Asn	Ala	Leu	His	Glu	Arg	Ala	Met	Arg	Arg	Arg	Ala	Gln	Leu
	530					535					540				
Ala	Asp	Ser	Phe	His	Leu	Gln	Gln	Phe	Phe	Arg	Asp	Ser	Asp	Glu	Leu
545					550					555					560
Lys	Ser	Trp	Val	Asn	Glu	Lys	Met	Lys	Thr	Ala	Thr	Asp	Glu	Ala	Tyr
				565					570					575	
Lys	Asp	Pro	Ser	Asn	Leu	Gln	Gly	Lys	Val	Gln	Lys	His	Gln	Ala	Phe
			580					585					590		
Glu	Ala	Glu	Leu	Ser	Ala	Asn	Gln	Ser	Arg	Ile	Asp	Ala	Leu	Glu	Lys
		595					600					605			
Ala	Gly	Gln	Lys	Leu	Ile	Asp	Val	Asn	His	Tyr	Ala	Lys	Asp	Glu	Val
	610					615					620				
Ala	Ala	Arg	Met	Asn	Glu	Val	Ile	Ser	Leu	Trp	Lys	Lys	Leu	Leu	Glu
625					630					635					640
Ala	Thr	Glu	Leu	Lys	Gly	Ile	Lys	Leu	Arg	Glu	Ala	Asn	Gln	Gln	Gln
				645					650					655	
Gln	Phe	Asn	Arg	Asn	Val	Glu	Asp	Ile	Glu	Leu	Trp	Leu	Tyr	Glu	Val
			660					665					670		
Glu	Gly	His	Leu	Ala	Ser	Asp	Asp	Tyr	Gly	Lys	Asp	Leu	Thr	Asn	Val
		675					680					685			
Gln	Asn	Leu	Gln	Lys	Lys	His	Ala	Leu	Leu	Glu	Ala	Asp	Val	Ala	Ala
	690					695					700				
His	Gln	Asp	Arg	Ile	Asp	Gly	Val	Thr	Ile	Gln	Ala	Arg	Gln	Phe	Gln
705					710					715					720
Asp	Ala	Gly	His	Phe	Asp	Ala	Glu	Asn	Ile	Lys	Lys	Lys	Gln	Glu	Ala
				725					730					735	

Leu Val Ala Arg Tyr Glu Ala Leu Lys Glu Pro Met Val Ala Arg Lys
 740 745 750
 Gln Lys Leu Ala Asp Ser Leu Arg Leu Gln Gln Leu Phe Arg Asp Val
 755 760 765
 Glu Asp Glu Glu Thr Trp Ile Arg Glu Lys Glu Pro Ile Ala Ala Ser
 770 775 780
 Thr Asn Arg Gly Lys Asp Leu Ile Gly Val Gln Asn Leu Leu Lys Lys
 785 790 795 800
 His Gln Ala Leu Gln Ala Glu Ile Ala Gly His Glu Pro Arg Ile Lys
 805 810 815
 Ala Val Thr Gln Lys Gly Asn Ala Met Val Glu Glu Gly His Phe Ala
 820 825 830
 Ala Glu Asp Val Lys Ala Lys Leu His Glu Leu Asn Gln Lys Trp Glu
 835 840 845
 Ala Leu Lys Ala Lys Ala Ser Gln Arg Arg Gln Asp Leu Glu Asp Ser
 850 855 860
 Leu Gln Ala Gln Gln Tyr Phe Ala Asp Ala Asn Glu Ala Glu Ser Trp
 865 870 875 880
 Met Arg Glu Lys Glu Pro Ile Val Gly Ser Thr Asp Tyr Gly Lys Asp
 885 890 895
 Glu Asp Ser Ala Glu Ala Leu Leu Lys Lys His Glu Ala Leu Met Ser
 900 905 910
 Asp Leu Ser Ala Tyr Gly Ser Ser Ile Gln Ala Leu Arg Glu Gln Ala
 915 920 925
 Gln Ser Cys Arg Gln Gln Val Ala Pro Thr Asp Asp Glu Thr Gly Lys
 930 935 940
 Glu Leu Val Leu Ala Leu Tyr Asp Tyr Gln Glu Lys Ser Pro Arg Glu
 945 950 955 960
 Val Thr Met Lys Lys Gly Asp Ile Leu Thr Leu Leu Asn Ser Thr Asn
 965 970 975
 Lys Asp Trp Trp Lys Val Glu Val Asn Asp Arg Gln Gly Phe Val Pro
 980 985 990
 Ala Ala Tyr Val Lys Lys Leu Asp Pro Ala Gln Ser Ala Ser Arg Glu
 995 1000 1005
 Asn Leu Leu Glu Glu Gln Gly Ser Ile Ala Leu Arg Gln Glu Gln Ile
 1010 1015 1020
 Asp Asn Gln Thr Arg Ile Thr Lys Glu Ala Gly Ser Val Ser Leu Arg
 1025 1030 1035 1040
 Met Lys Gln Val Glu Glu Leu Tyr His Ser Leu Leu Glu Leu Gly Glu
 1045 1050 1055
 Lys Arg Lys Gly Met Leu Glu Lys Ser Cys Lys Lys Phe Met Leu Phe
 1060 1065 1070

Arg	Glu	Ala	Asn	Glu	Leu	Gln	Gln	Trp	Ile	Asn	Glu	Lys	Glu	Ala	Ala
		1075					1080					1085			
Leu	Thr	Ser	Glu	Glu	Val	Gly	Ala	Asp	Leu	Glu	Gln	Val	Glu	Val	Leu
		1090				1095					1100				
Gln	Lys	Lys	Phe	Asp	Asp	Phe	Gln	Lys	Asp	Leu	Lys	Ala	Asn	Glu	Ser
1105					1110					1115					1120
Arg	Leu	Lys	Asp	Ile	Asn	Lys	Val	Ala	Glu	Asp	Leu	Glu	Ser	Glu	Gly
				1125					1130					1135	
Leu	Met	Ala	Glu	Glu	Val	Gln	Ala	Val	Gln	Gln	Gln	Glu	Val	Tyr	
			1140					1145					1150		

APPENDIX I
PENDING CLAIMS AS AMENDED IN THIS COMMUNICATION

The following is a list of the claims as they would appear following entry of this amendment.

15. (Once amended) A method for assessing overcoming synaptic vesicle glutamate uptake inhibition activity, comprising:

- a) providing:
 - i) synaptic vesicles,
 - ii) a composition comprising purified fodrin fragment having glutamate uptake inhibition activity, said fragment having an N-terminus and a C-terminus, and
 - iii) candidate compound; and
- b) combining said candidate compound with said synaptic vesicles and said fragment such that the effect of said candidate compound on glutamate uptake by said synaptic vesicles can be assessed.

16. The method of Claim 15, wherein said purified fragment comprises IPF α .

17. The method of Claim 15, wherein said purified fragment comprises IPF β .

18. The method of Claim 15, wherein said purified fragment comprises IPF γ .

19. (Once Amended) A method for assessing overcoming synaptic vesicle glutamate uptake inhibition activity, comprising:

- a) providing:
 - i) synaptic vesicles,
 - ii) a composition comprising purified fragment of IPF having synaptic vesicle glutamate uptake inhibition activity, said fragment having an N-terminus and a C-terminus, and
 - iii) candidate compound; and

b) combining said candidate compound with said synaptic vesicles and said purified fragment such that the effect of said candidate compound on said fragment's effect on glutamate uptake by said synaptic vesicles can be assessed.

20. The method of Claim 19, wherein said fragment comprises a fragment of IPF α .

21. The method of Claim 19, wherein said fragment comprises a fragment of IPF β .

22. The method of Claim 19, wherein said fragment comprises a fragment of IPF γ .

23. (New) The method of Claim 15, wherein said N-terminus is Tyr²⁶ of fodrin.

24. (New) The method of Claim 15, wherein said purified fragment comprises a fragment of IPF α .

25. (New) The method of Claim 19, wherein said N-terminus is Tyr²⁶ of fodrin.

26. (New) The method of Claim 19, wherein said purified fragment comprises a fragment of IPF α .

27. (New) A method for assessing overcoming synaptic vesicle glutamate uptake inhibition activity, comprising:

- a) providing:
 - i) synaptic vesicles,
 - ii) a composition comprising preferred fragment of fodrin having glutamate uptake inhibition activity, said fragment having an N-terminus and a C-terminus, wherein said purified fragment comprises a peptide having the amino

acid sequence EAALTSEEVG within 150 amino acids of the C-terminus of the peptide, and

- iii) candidate compound; and
- b) combining said candidate compound with said synaptic vesicles and said fragment such that the effect of said candidate compound on glutamate uptake by said synaptic vesicles can be assessed.

28. (New) A method for assessing overcoming synaptic vesicle glutamate uptake inhibition activity, comprising:

- a) providing:
 - i) synaptic vesicles,
 - ii) a composition comprising purified peptide having glutamate uptake inhibition activity with an N-terminus sequence comprising the amino acid sequence YHRFK, and
 - iii) candidate compound; and
- b) combining said candidate compound with said synaptic vesicles and said fragment such that the effect of said candidate compound on glutamate uptake by said synaptic vesicles can be assessed.

29. (New) The method of Claim 28, wherein said purified peptide has an N-terminus comprising the amino acid sequence YHRFKELSTL.

30. (New) The method of Claim 29, wherein said purified peptide has an N-terminus comprising the amino acid sequence YHRFKELSTLRRQKLEDSYR.